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# Journal Pre-proof

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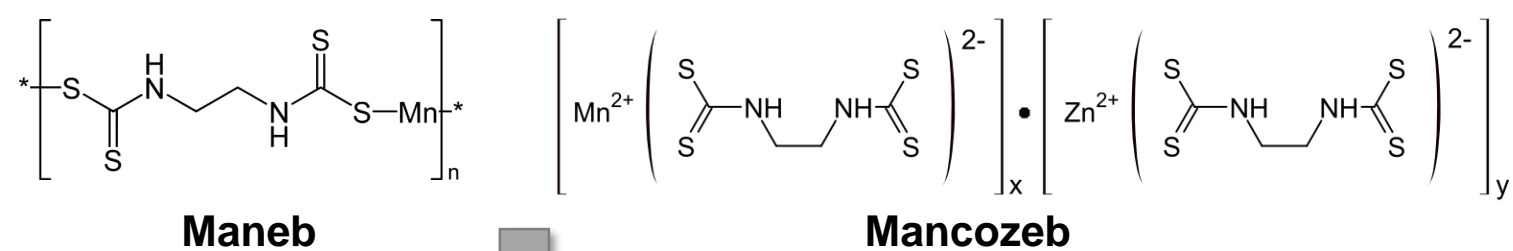


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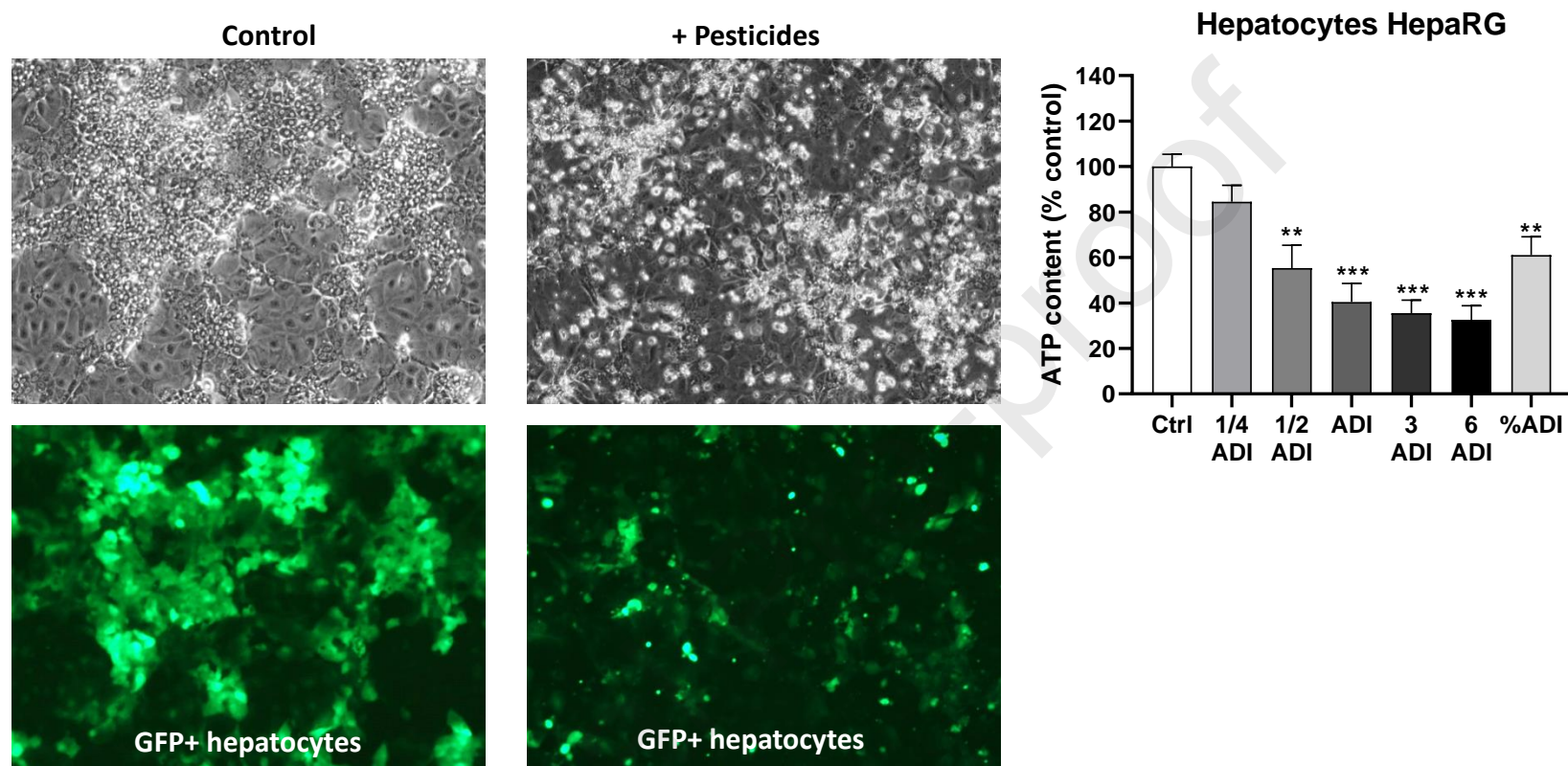
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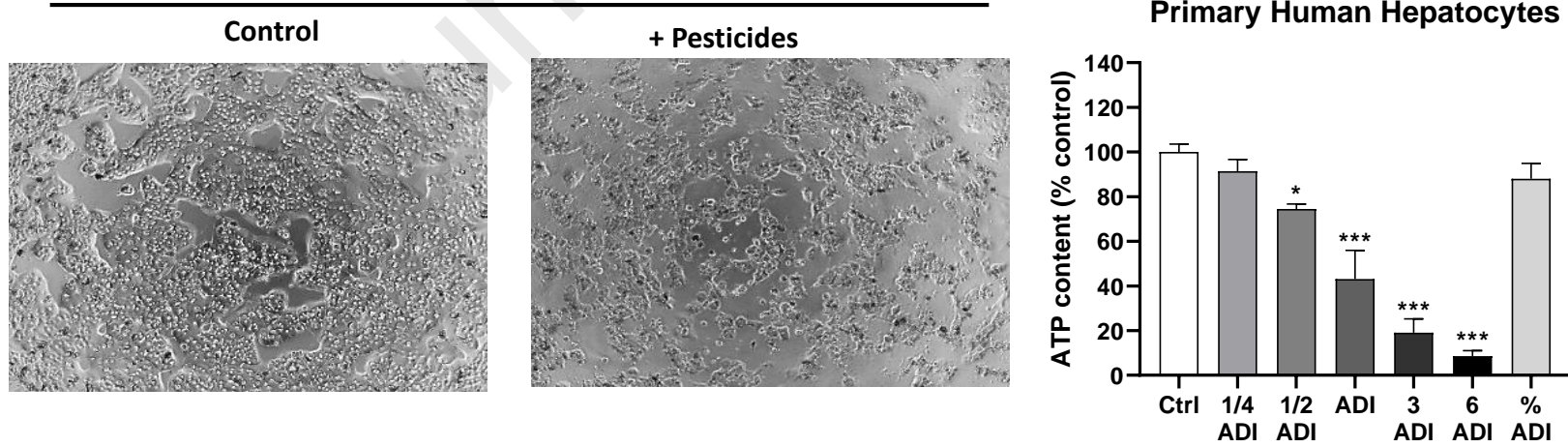
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Hepatocytes HepaRG



Human Hepatocytes



1 **Low concentrations of ethylene bisdithiocarbamate pesticides maneb and**  
2 **mancozeb impair manganese and zinc homeostasis to induce oxidative stress**  
3 **and caspase-dependent apoptosis in human hepatocytes**

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21  
22 **Keywords** : maneb ; mancozeb ; manganese ; zinc ; oxidative stress ; hepatocytes.

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31 **Abbreviations:** DTCs: Dithiocarbamates, CYP450: cytochrome P450; DMSO: dimethyl  
32 sulfoxide ; XMEs: xenobiotic metabolizing enzymes ; HPLC-UV: High Pressure Liquid  
33 Chromatography-Ultra Violet ; LC-MS: Liquid-Chromatography-mass spectrometry ; ROS:  
34 Reactive Oxygen Species.

35

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## ABSTRACT

The worldwide and intensive use of phytosanitary compounds results in environmental and food contamination by chemical residues. Human exposure to multiple pesticide residues is a major health issue. Considering that the liver is not only the main organ for metabolizing pesticides but also a major target of toxicities induced by xenobiotics, we studied the effects of a mixture of 7 pesticides (chlorpyrifos-ethyl, dimethoate, diazinon, iprodione, imazalil, maneb, mancozeb) often detected in food samples. Effects of the mixture was investigated using metabolically competent HepaRG cells and human hepatocytes in primary culture. We report the strong cytotoxicity of the pesticide mixture towards hepatocytes-like HepaRG cells and human hepatocytes upon acute and chronic exposures at low concentrations extrapolated from the Acceptable Daily Intake (ADI) of each compound. Unexpectedly, we demonstrated that the manganese (Mn)-containing dithiocarbamates (DTCs) maneb and mancozeb were solely responsible for the cytotoxicity induced by the mixture. The mechanism of cell death involved the induction of oxidative stress, which led to cell death by intrinsic apoptosis involving caspases 3 and 9. Importantly, this cytotoxic effect was found only in cells metabolizing these pesticides. Herein, we unveil a novel mechanism of toxicity of the Mn-containing DTCs maneb and mancozeb through their metabolization in hepatocytes generating the main metabolite ethylene thiourea (ETU) and the release of Mn leading to intracellular Mn overload and depletion in zinc (Zn). Alteration of the Mn and Zn homeostasis provokes the oxidative stress and the induction of apoptosis, which can be prevented by Zn supplementation. Our data demonstrate the hepatotoxicity of Mn-containing fungicides at very low doses and unveil their adverse effect in disrupting Mn and Zn homeostasis and triggering oxidative stress in human hepatocytes.

## 91 **1. Introduction**

92 Pesticides remain extensively used in agricultural practices despite the well-documented risks  
93 for the human health (Alvanja, 2009 ; Deziel et al., 2017). Occupational exposures to pesticides  
94 have been clearly correlated to higher risks of a wide range of pathologies including  
95 developmental abnormalities (Rauh et al., 2012), Parkinson disease (Brouwer et al., 2017 ;  
96 Pouchieu et al., 2018), hematological disorders (Merhi et al., 2007 ; Jin et al., 2014), solid  
97 tumors (Sabarwal et al., 2018; Lerro et al., 2021) and leukemia (Ferri et al., 2017 ; Foucault et  
98 al., 2021a). Higher occurrences of pesticide-dependent diseases are not restricted to  
99 occupational exposure but are also documented in populations living in agricultural areas of  
100 intensive spreading via the drifting of pesticides in the air and house dust contamination (Deziel  
101 et al., 2017).

102 The intensive use of pesticides contributes to the so-called diffuse pollution (Mghirbi et al.,  
103 2018) with accumulation of chemicals in the upper layers of soil resulting from atmospheric  
104 deposition (Mali et al., 2023) and land application (Ritter et al., 2002). In addition, the soil  
105 contamination and the direct spreading of pesticides on crops result in the presence of chemical  
106 residues in agricultural products (Czaja et al., 2020). Regulatory authorities such as the Food  
107 and Drug Administration (US FDA), the European Food Safety Authority (EFSA), World  
108 Health Organization (WHO) and the Organization for Economic Co-operation and  
109 Development (OECD) defined residues as the mixture of active substances and degradation  
110 products and established a threshold indicator of acceptable contamination, the Maximum  
111 Residue Levels (MRLs), corresponding to the highest levels of pesticide residues legally  
112 tolerated in agricultural commodities when pesticides are applied correctly and the lowest  
113 exposure in order to protect vulnerable consumers.

114 These agencies also monitor the presence of residues in food samples (OECD 2009 ; EFSA  
115 2017 ; US FDA 2019) to estimate the dietary risk assessment of general population to pesticide  
116 exposures by calculating the Theoretical Maximum Daily Intake (TMDI) (EFSA 2017 ; EFSA  
117 2019). In the past decade, the presence of at least one chemical contaminant in nearly 50% of  
118 food products and multiple residues in 25% of the samples was reported (OECD 2009 ; EFSA  
119 2017 ; US FDA 2019). In most cases, the amounts of residues in human foods were compliant  
120 with MRLs, however, for 10% of these commodities, contamination levels exceeded those  
121 maximum acceptable levels. Considering the large diversity of active substances used in  
122 agricultural practice, it is practically impossible to monitor all active substances and their  
123 metabolites. For instance, the large family of dithiocarbamates (DTCs) fungicides (Ajiboye et



124 al. 2022), including maneb and mancozeb used worldwide, are difficult to identify individually  
125 by analytical procedures and only total DTC concentrations can be measured in water (Bristeau  
126 and Ghestem, 2018 ; Bristeau and Amalric, 2021). Nevertheless, the assessment of  
127 contamination levels in commodities remains poorly characterized and exposure to DTCs in  
128 humans relies only on the detection of their main metabolite, ethylene thiourea (ETU), in urine  
129 (Stadler et al., 2022).

130 The approval of pesticides is based on individual toxicology studies of each active substance,  
131 which allow to define criteria of risk assessment. The Acceptable daily intake (ADI), expressed  
132 in mg of chemical per kilogram of body weight per day (mg/kg.bw/day), is defined as the  
133 maximum amount of a chemical that can be ingested daily over a lifetime with no appreciable  
134 health risk (Gray 2023).

135 The ADI, which is a theoretical safety threshold for human health, is calculated from the No  
136 observable Adverse Effect Level (NoAEL) determined in animal models applying a corrective  
137 factor to account for intra-species and intra-individual variations (Dorne, 2010). Nevertheless,  
138 ADI does not consider potential cumulative effects of pesticide mixtures while regulatory  
139 agencies recognized that consumers are exposed to multiple residues present in food for long  
140 periods of times, which could lead to cumulative and delayed effects on human health. In this  
141 context, it is necessary to test low doses of mixtures of pesticides belonging to different families  
142 with similar or different mechanisms of action to better assess the possible existence of a risk  
143 to human health. Exposures to low doses of pesticide mixtures could lead to insidious  
144 synergistic effects significantly different from those observed if summing the effect of each  
145 pesticide evaluated individually.

146 In order to extend knowledge on cellular alterations induced by pesticide mixtures, the  
147 consortium project entitled “Pesticides and Tumor Niches” (Cancéropole Grand-Ouest, France)  
148 studied the adverse effects of a mixture of 7 pesticides from different classes of chemicals  
149 (chlorpyrifos-ethyl, dimethoate, diazinon, iprodione, imazalil, maneb, mancozeb) often  
150 detected in food samples (EFSA, 2017). These compounds were used at low concentrations  
151 extrapolated from the ADI dose for each pesticide in various *in vitro* cell models. During this  
152 project, it was shown that the pesticide mixture triggered the senescence of bone marrow  
153 mesenchymal stromal cells (BM-MSCs), their differentiation in adipocytes (Hochane et al.,  
154 2017) and the deterioration of their immunosuppressive properties (Leveque et al., 2019). These  
155 pesticides also altered the BM-MSC capacity to support primitive hematopoiesis by monitoring  
156 *in vitro* expansion of human normal BM CD34<sup>+</sup> progenitors co-cultured with pesticides-

157 modified BM-MSCs (Foucault et al., 2021b). In this last study, the manganese (Mn)-containing  
158 DTCs, maneb and mancozeb were shown to significantly contribute to the decrease in the  
159 CD34+ cell expansion (Foucault et al., 2021b). Interestingly, similar alterations have been  
160 described in BM-MSCs of patients suffering from myelodysplastic syndromes supporting the  
161 hypothesis that exposure to pesticides could favor the appearance of pre-leukemic states in the  
162 elderly (Foucault et al., 2021a).

163 Most pesticides are metabolized in the liver by phase I and II xenobiotic enzymes (XMEs) to  
164 produce hydrophilic products more easily eliminated in urine and bile (Jandacek and Tso, 2007  
165 ; Klimowska et al., 2020 ; Kernalléguen et al., 2022). The liver is also a major target for  
166 toxicities triggered by xenobiotics themselves and/or their reactive metabolites. In the present  
167 study, we evaluated the effects of the same pesticide mixture and the 7 pesticides tested  
168 individually on metabolically competent hepatocytes *in vitro* at concentrations that previously  
169 showed effects of BM-MSCs (Hochane et al., 2017 ; Leveque et al., 2019 ; Foucault et al.,  
170 2021b). We report that the pesticide mixture induced a strong cytotoxicity in cultures of  
171 hepatocyte-like HepaRG cells and human hepatocytes in acute and chronic exposures at low  
172 concentrations extrapolated from the ADI. In addition, we demonstrated that the metal-  
173 containing DTCs maneb and mancozeb were solely responsible for the cytotoxicity induced by  
174 the mixture but only in cells metabolizing these fungicides through the intracellular  
175 accumulation of manganese (Mn) and reduction in zinc (Zn) contents, which triggered oxidative  
176 stress and apoptosis. Together, our data unveil a novel mechanism of hepatotoxicity of Mn-  
177 containing fungicides and emphasize the crucial role of the Mn and Zn homeostasis to regulate  
178 ROS production and cell fate in human hepatocytes.

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## 190 2. Materials and methods

### 191 2.1. Pesticides and preparation of the %ADI and ADI mixtures.

192 Chlorpyrifos, dimethoate, diazinon, imazalil, iprodione, maneb, mancozeb, zineb Pestanal<sup>TM</sup>  
193 analytical standards, ethylenethiourea (45531), ethyleneurea (31534) and ethylenediamine  
194 (41008) were purchased from Sigma Aldrich (St. Louis, MO, USA). Chemical structures are  
195 provided in **Supporting Information 1**.

196 The concentrations of pesticides used to treat the cells (**Table 1**) were extrapolated from the  
197 ADI and high Nutritional Daily Intake (hNDI) values as previously described (Hochane et al.,  
198 2017 ; Leveque et al., 2019). The ADI value represents the threshold of safety in humans for  
199 lifetime exposure, specified by the WHO and the European Commission. The hNDI  
200 corresponds to the real exposure of the French population of all ages for each pesticide and  
201 calculated from the MRLs in food commodities established by EFSA (EFSA, 2010). The  
202 method used by the EFSA to determine chronic exposure is comparable to the calculation of  
203 the theoretical maximum daily intake (TMDI) according to the following equation:

$$204 \quad \text{TMDI} = \sum \text{MRL}_i \times F_i$$

205 MRL<sub>i</sub>: Maximum residue level for food commodity I ; F<sub>i</sub>: Food consumption of food  
206 commodity i.

207 In order to introduce a more realistic estimation of the exposure to a pesticide and to calculate  
208 hDNI, the EFSA replaced the MRL<sub>i</sub> values by the residue concentrations obtained on analyzed  
209 food samples. The results were reported for each pesticide in 3 groups (infants, toddlers, and  
210 general population) and for 27 different diets (EFSA, 2010 ; EFSA, 2019). The hNDI value  
211 corresponding to the highest estimated exposure for each pesticide is expressed as percent of  
212 the ADI (%ADI, **Table 1**) reported for each diet. We calculated an average value of %ADI  
213 from all diets that we converted to mg/kg.bw/day considering the ADI value for each pesticide.  
214 Then, values of ADI and %ADI (mg/kg.bw/day) for each pesticide were converted to g/L and  
215 then in mol/L for *in vitro* studies (**Table 1**). We considered a total absorption of these ingested  
216 amounts and their dilution in 5 L of blood in a subject of 60 kg, in order to obtain the blood  
217 concentration (mg/L and  $\mu\text{mol/L}$ ) to which the various organs could be theoretically exposed.  
218 Dimethyl-sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) was the solvent used to  
219 prepare stock solutions at 100mM for 6 pesticides except for the diazinon at 10mM. From these  
220 stock solutions, dilutions were performed for each pesticide in DMSO to prepare 2 intermediate  
221 mixtures: [%ADI]<sup>3</sup> and [6ADI]<sup>3</sup> mixtures corresponding to concentrations of pesticides 10<sup>3</sup>-

222 fold higher than %ADI and 6ADI final mixtures used in culture media, respectively. From these  
223 intermediate mixtures, dilutions were performed in culture media to obtain the final  
224 concentrations of exposure at  $\frac{1}{4}$ ADI  $\frac{1}{2}$ ADI, ADI, 3ADI, 6ADI or %ADI in cell models. Values  
225 for ADI and %ADI concentrations as well as the proportion of each pesticide in the mixtures  
226 are given in **Table 1**.

227

228

## 229 *2.2. Cell cultures.*

230 The human hepatocellular HepaRG progenitor cells actively proliferate at low density and are  
231 capable to differentiate into both biliary- and hepatocyte-like cells in appropriate culture  
232 conditions (Gripon et al., 2002 ; Cerec et al., 2007). Progenitor HepaRG cells were cultured as  
233 previously described (Cerec et al., 2007) in William's E Medium (Gibco, Thermo Fisher  
234 Scientific, Waltham, MA, USA) supplemented with Fetal Bovine Serum (FBS) 10% (Eurobio  
235 Scientific, Les Ulis, France. Batch S52446-2170, reference CVFSVF00-01, HyClone Foetal II  
236 batch AAF204244 reference SH30066.03, ratio 1-1), 1% glutamine (200mM), 100U/ml  
237 penicillin, 100 $\mu$ g/ml streptomycin, 5 $\mu$ g/ml insulin, and 50 $\mu$ M hydrocortisone hemisuccinate  
238 (Upjohn pharmacia). HepaRG cells were seeded at a density of  $2.6 \times 10^4$  cells/cm<sup>2</sup>. After 2  
239 weeks, cells were cultured in the same medium supplemented with 2% DMSO to obtain  
240 differentiated HepaRG cells including hepatocyte- and cholangiocyte-like cells (Cerec et al.,  
241 2007). In some experiments, hepatocyte- and cholangiocytes-like HepaRG cells were  
242 selectively detached using mild trypsinization with Trypsin 0.05% diluted in PBS (vol/vol) as  
243 previously described (Cerec et al., 2007 ; Laurent et al., 2013) and plated separately at high  
244 density ( $2.5 \times 10^5$  cells/cm<sup>2</sup>) to obtain cultures enriched in hepatocytes or cholangiocytes. Both  
245 hepatocyte- and cholangiocyte-like cells were cultured in medium supplemented with 2%  
246 DMSO.

247 Human liver biopsies and primary hepatocytes were obtained from the processing of biological  
248 samples through the Centre de Ressources Biologiques (CRB) Santé of Rennes (Institutional  
249 review board approval BB-0033-00,056) under French legal guidelines and fulfilled the  
250 requirements of the institutional ethics committee. Primary human hepatocytes (PHH) were  
251 seeded at high density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> and cultured in same medium than differentiated  
252 HepaRG hepatocytes. Culture procedures of human macrophages and BM-MSC, as well as rat  
253 liver epithelia cells SDVI, Huh7 and HepG2 hepatoma cells are described in **Supporting**  
254 **Information 1**.

255 *2.3. Treatments with pesticides, N-acetylcysteine, MnCl<sub>2</sub> and ZnCl<sub>2</sub>.*

256 Treatments of cells at the ADI and %ADI values (**Table 1**) resulted in exposures to pesticide  
257 concentrations ranging from 0.0078 to 2.262  $\mu\text{M}$  (ADI value) and 0.00286 to 0.73  $\mu\text{M}$  (%ADI  
258 value) with differences in the percentage of each pesticide in the two ADI and %ADI mixtures  
259 (**Table 1**). Control condition corresponded to cultures not treated by pesticides but exposed to  
260 the same concentration of DMSO used to dissolve the pesticides.

261 For differentiated HepaRG cells and human hepatocytes, DMSO in the culture medium was  
262 adjusted at 2% (vol/vol), which corresponded to the culture medium used routinely for keeping  
263 these cells differentiated. For progenitor HepaRG cells and non-hepatic cells, DMSO  
264 concentration was 0.1%.

265 For chronic exposures of progenitor HepaRG cells, one day after cell seeding at low density,  
266 proliferating progenitor cells were treated (day 1), then every 2 or 3 days at each renewal of  
267 culture medium (3 times a week). In these experiments, cell viability assays (see paragraph 2.4)  
268 were performed at day 7 (after 3 treatments), at day 14 (6 treatments), at day 21 (9 treatments),  
269 and at day 30 (12 treatments). For acute exposures, HepaRG cells were first differentiated over  
270 30 days without exposure to pesticides, then differentiated cells were exposed to a single  
271 treatment for 48 hours (48h) prior to cell viability assay. For enriched hepatocyte- and  
272 cholangiocyte-like HepaRG cell cultures prepared by selective detachment (paragraph 2.2),  
273 differentiated cells when plated for two days, then subjected to single treatment for 48h.  
274 Pictorial charts depict the experimental design of both chronic and acute exposures of HepaRG  
275 cells with pesticides in the corresponding figures.

276 Similar chronic treatments with  $\text{MnCl}_2$  and  $\text{ZnCl}_2$  were also performed only in differentiated  
277 HepaRG cells for 48h. In order to compare the effects of maneb, mancozeb, zineb, Mn and Zn  
278 on cell viability, 7  $\mu\text{M}$  of manganese chloride ( $\text{MnCl}_2$ ) or zinc chloride ( $\text{ZnCl}_2$ ) were used,  
279 which corresponded to the same concentration in Mn or Zn brought by the 2 DTCs maneb and  
280 zineb at 3ADI concentration, respectively.

281 For human hepatocytes in primary cultures and all the other cell types, acute exposures (single  
282 treatment for 48h) were performed 2 days after plating.

283 N-acetylcysteine (NAC, Sigma Aldrich, St. Louis, MO, USA) at 20mM was used to neutralize  
284 ROS by treating cells for 2h prior to and during the acute exposure to pesticides in differentiated  
285 HepaRG cells. Treatments with 10-fold excess in  $\text{ZnCl}_2$  at 70 $\mu\text{M}$  were also performed during  
286 pesticide (3ADI) or  $\text{MnCl}_2$  (7 $\mu\text{M}$ ) exposures.

287

288 *2.4. Cell viability, apoptosis assessment and genotoxicity.*

289 Cell viability was assessed by measuring the relative intracellular ATP content using CellTiter-  
290 Glo Luminescent Cell viability assay (Promega, Madison, WI, USA – Charbonnières Les Bains,  
291 France). After treatment with pesticides, cells were incubated with the CellTiter-Glo reagent  
292 for 10 minutes (min). Lysates were transferred to an opaque multi-well plate and luminescent  
293 signals were quantified at 540nm using the Polarstar Omega microplate reader (BMG Labtech,  
294 Champigny sur Marne, France). Cell viabilities in treated cells were expressed as the percentage  
295 of the luminescent values obtained in untreated cells, which was arbitrary set as 100%. Loss of  
296 plasma membrane integrity and cytotoxicity during treatments were assessed by measuring the  
297 lactate dehydrogenase (LDH) release in the culture medium using ThermoScientific™ Pierce™  
298 LDH Cytotoxicity Assay Kit (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA)  
299 according to manufacturer's instructions.

300 Caspases activities were quantified after 24 or 48h of treatment for HepaRG cells and PHH,  
301 respectively. Cells were lysed with the “caspase-activity” buffer containing PIPES (20mM: pH  
302 7.2), NaCl (100mM), EDTA (1mM), CHAPS (0.1%), sucrose (10%). Lysates were  
303 homogenized using sonication for 10 seconds twice. Then, 50µg proteins were added to the  
304 caspase-activity buffer supplemented with 10mM DTT and 80µM of substrates Ac-DEVD-  
305 AMC (ALX 260-031), Ac-IEPD-AMC (reference BML-P134) and Ac-LEHD-AMC (reference  
306 ALX 260-080) purchased from Enzo Life Science (Farmingdale, NY, USA) to measure caspase  
307 3, 8 and 9 activities, respectively. The luminescence was measured using the Polarstar Omega  
308 microplate reader (BMG Labtech, Champigny sur Marne, France) with excitation wavelengths  
309 at 380 and emission at 440nm each 10 min during a 2h-time course.

310 The  $\gamma$ -H2AX test was performed to assess genotoxicity. Briefly, cells were rinsed with  
311 phosphate-buffered saline (PBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), fixed  
312 with 4% formaldehyde in PBS for 15 min at room temperature before permeabilization using  
313 0.1% saponin in PBS. Nonspecific binding was blocked for 15 min by incubating the cells with  
314 2 % FBS in PBS. Then, the cells were incubated with rabbit polyclonal anti-phospho-H2AX  
315 antibody (reference ab88671, Abcam, Cambridge, United Kingdom) diluted 1/500 in 2 % FBS-  
316 PBS solution for 1h at room temperature. Following washes with PBS, Dylight 550-labeled  
317 secondary antibody (reference 96892, Abcam, Cambridge, United Kingdom) in 2 % FBS-PBS  
318 solution containing Hoechst 33342 dye (1µg/mL) was incubated for 45 min. After washing, the  
319  $\gamma$ -H2AX positive nuclei were observed and ten pictures per well were analyzed corresponding  
320 to at least 1000 cells scored.

321 *2.5. Detection of reactive oxygen species (ROS).*

322 Cell-permeant CellROX Orange (reference C10493), 2',7'-dichlorodihydrofluorescein diacetate  
323 (H<sub>2</sub>DCFDA, reference D399) and MitoSOX (reference M36008) molecular probes, purchased  
324 from Invitrogen™ (Thermo Fisher Scientific, Waltham, MA, USA) were used to assess ROS  
325 generation. These probes are recommended to measure general oxidative stress (CellROX and  
326 H<sub>2</sub>DCFDA) and mitochondrial superoxide (MitoSOX). Cells were exposed to pesticides for  
327 24h, then washed with prewarmed (37°C) Hanks' Balanced Salt Solution (HBSS, (Gibco,  
328 Thermo Fisher Scientific, Waltham, MA, USA) and incubated with CellROX Orange (500nM)  
329 or MitoSOX (2μM) diluted in HBSS for 30 min at 37°C, 5% CO<sub>2</sub> protected from light. Then,  
330 cells were rinsed with warm HBSS. For MitoSOX probe, fluorescence intensity was measured  
331 using the Polarstar Omega microplate reader (BMG Labtech, Champigny sur Marne, France)  
332 with excitation/emission wavelengths and 520/590nm. Fluorescence intensity values were  
333 normalized to amounts of total proteins. For CellROX Orange probe, the fluorescence intensity  
334 of at least 5,000 cells was analyzed with a Becton Dickinson (Franklin Lakes, NJ, USA –  
335 Grenoble, France) LSRFortessa™ X-20 (cytometry core facility of the Biology and Health  
336 Federative research structure Biosit, Rennes, France). Data were expressed as the percentage of  
337 fluorescence signal measured in untreated control cells, which was arbitrarily set as 100%.

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340 *2.6. Measurement of mitochondrial respiration and oxidative phosphorylation (OXPHOS)*  
341 *complex enzyme activities.*

342 Parameters of mitochondrial respiration were analyzed using XF Cell Mito Stress Test Kits and  
343 Agilent Seahorse XFe24 analyzer (Agilent Technologies, Santa Clara, CA, USA) according to  
344 manufacturer's instructions. Briefly, after 16h of exposure to pesticides, HepaRG cells were  
345 incubated with Seahorse XF DMEM Medium (pH 7.4) at 37°C for 1 hour. Then, Seahorse XF  
346 DMEM Medium (pH 7.4) was replaced by the same medium containing L-glutamine (2mM),  
347 glucose (10mM) and pyruvate (1mM). HepaRG cells were transferred in Agilent Seahorse  
348 XFe24 analyzer for assessment of mitochondrial respiration. Oxygen consumption rates  
349 corresponding to basal respiration, maximal respiration, ATP production and proton leak were  
350 normalized by fluorescence intensity of Hoechst 33342 dye (reference 62249, Thermo Fisher  
351 Scientific, Waltham, MA, USA) measured with the Polarstar Omega microplate reader (BMG  
352 Labtech, Champigny sur Marne, France) with excitation/emission wavelengths at  
353 355nm/460nm.

354 In order to measure OXPHOS complex enzyme activities mitochondria were first isolated from  
355 HepaRG cells treated for 16h with maneb at 6ADI, MnCl<sub>2</sub> at 14μM or control solvent vehicle.  
356 Briefly, cells were harvested and washed with cold PBS, then cells were centrifuged and pellets  
357 were frozen in liquid nitrogen. Cells were resuspended within isolation buffer pH 7.2 containing  
358 mannitol (220mM), sucrose (75mM), Trizma (10mM) and EDTA (1mM). After 3 min frozen  
359 in liquid nitrogen, cells were thawed at 37°C, centrifuged 1min at 20,000g and finally  
360 resuspended in the isolation buffer. For complexes I and V and citrate synthase cells were  
361 sonicated (6 times for 5 s).

362 The activities of the 5 mitochondrial OXPHOS complex enzymes were then measured at 37°C  
363 on a UVmc<sup>2</sup> spectrophotometer (SAFAS Monaco, 98000 Monaco) as previously described  
364 (Chrétien et al., 2004 ; Medja et al., 2009) with specific buffer and reagents for each complex  
365 (**Supporting Information 1**). Absorbance changes due to the respective substrate conversions  
366 were analyzed at, 600nm for complex I and II, 550nm for complex III and IV, 340nm for  
367 complex V and 412nm for citrate synthase. Enzymes activities of all complexes were expressed  
368 as nmol substrate/min/10<sup>6</sup>cells using the Beer Lambert's law and were normalized to the citrate  
369 synthase activity.

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372 *2.7. Studies of mancozeb metabolism by High pressure liquid chromatography (HPLC) and*  
373 *liquid chromatography coupled to mass spectrometry (LC-MS/MS).*

374 The HPLC analysis was performed with the Agilent HPLC 1260 Infinity II (Agilent  
375 Technologies, Santa Clara, CA, USA) with the column Accucore PFP (reference 17426-  
376 153030, Thermo Fisher Scientific, Waltham, MA, USA) coupled with a UV detector. Cells  
377 were washed with PBS and incubated with phenol red-free William's E Medium containing  
378 100μM, maneb or mancozeb. After incubations of cells for 3, 8 or 24h, the medium was  
379 collected and centrifuged 15 min at 20,000g at 4°C. 50μL of supernatant was injected in HPLC  
380 system. A gradient of acetonitrile and 0.1% acetic acid was used during the run at a flow rate  
381 of 0.500 mL/min. The run starts at 100% of acetic acid and decrease to 10% in 20 min. After 2  
382 min at 10% acetic acid and 90% acetonitrile, the gradient returns to 100% acetic acid in 5 min.  
383 The wavelengths 247 and 285 nm were used to detect the metabolites of mancozeb, and the  
384 mancozeb, respectively. The peaks of mancozeb and ETU were identified by comparing the  
385 chromatograms of culture media containing or not these compounds and using analytical  
386 standards.



387 The analysis was performed by LC-MS/MS detection with direct injection using Xevo-TQD ®  
388 triple quadrupole UPLC/MSMS (Waters, Milford, MA, USA) in MRM (Multiple Reaction  
389 Monitoring) mode. The mass spectrometer was operated in the positive and negative electron  
390 ionization modes for identification of the unknown compound resulting from mancozeb and  
391  $\text{MnCl}_2$  exposures while the mode of ionization positive electrospray was selected for  
392 quantification of mancozeb metabolites. The injection of analytical standards allowed to define  
393 the analytical parameters of the 8 metabolites and active substance (**Supporting Information**  
394 **2**) and to estimate their quantification limits. To investigate the linearity of the method, 9  
395 concentrations (0.01 to 10  $\mu\text{M}$  in a mixture water/William's E Medium 50/50 v/v) were used  
396 and a quadratic model was for all compounds except ETU for which a cubic model gave a better  
397 fit. The samples were analyzed directly or after dilution with a mixture water/William's E  
398 Medium (50/50 v/v) when appropriate. A control (calibration point at 5  $\mu\text{M}$ ) was injected every  
399 15 samples to check the calibration accuracy. The samples to be analyzed were culture media  
400 containing or not mancozeb and incubated or not with HepaRG cells.

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#### 403 *2.8. Mn and Zn quantification by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).*

404 HepaRG cells were treated during 16h with maneb at 3ADI concentration,  $\text{MnCl}_2$  at 7  $\mu\text{M}$  or  
405 vehicle solvent (DMSO) as negative control. Then, cells were collected using trypsin (0.05%)  
406 before centrifugation. Cell pellets were washed twice in PBS then frozen at  $-80^\circ\text{C}$ . Cells or  
407 purified mitochondria (see section 2.5) were desiccated overnight at  $120^\circ\text{C}$ . Dried tissues were  
408 weighed and mineralized by nitric acid solution in teflon PFA-lined digestion vessels. Acid  
409 digestion was carried out at  $180^\circ\text{C}$  using ultrapure concentrated nitric acid ( $\text{HNO}_3$ ) solution  
410 (69%) (reference 11984091, Fisher Chemical Optima Grade, Thermo Fisher Scientific,  
411 Waltham, MA, USA) in a microwave oven device (Mars 6 microwave digestion system, CEM  
412 Corporation, Matthews, NC, USA – CEM  $\mu\text{Waves}$ , Orsay, France). The elements studied were  
413  $^{55}\text{Mn}$  and  $^{66}\text{Zn}$ . ICP-MS analyses were carried out using an X-Series II from Thermo Scientific  
414 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with collision cell technology  
415 (Platform AEM2, Biochemical Laboratory, Rennes Hospital). The source of plasma was argon  
416 (purity degree > 99.99%). The collision/reaction cell used was pressurized with a mixture of  
417 helium (93%) and hydrogen (7%); argon and hydrogen were provided by Messer Group (Bad  
418 Soden, Germany). Ultra-pure water was provided from Millipore Direct-Q 3 water station. The  
419 rhodium was used as internal standard (Thermo Fisher Scientific, Waltham, MA, USA).

420 Calibration ranges were realized using a multi-element solution (*PlasmaCal* Standards, SCP  
421 Science, Clark Graham Baie D'Urfé, Quebec, Canada). The performance was calibrated using  
422 multi-element solutions, tune F (reference NC0099423), and tune A (NC9063415) (Thermo  
423 Fisher Scientific, Waltham, MA, USA). The certified reference biological material was bovine  
424 liver ZC71001 obtained from NCS Testing Technology Company (Beijing, China).

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### 427 *2.9. RNA and protein expression studies.*

428 RNA purification and reverse transcription were performed using Nucleospin RNA (Fisher  
429 Scientific ref: 740955) and High capacity cDNA reverse transcription kit (Applied Biosystems).  
430 Quantitative PCR was performed with Sybr Green PCR Master Mix (Applied Biosystems,  
431 Thermo Fisher Scientific, Waltham, MA, USA) on ABI PRISM 7900HT instrument. The  
432 primers were purchased from Eurogentec S.A. (Seraing, Belgium) and the sequences are  
433 provided in **Supporting Information 1**.

434 For immunoblotting, culture medium was removed and cells were washed once with cold PBS  
435 and lysed in 50 mM HEPES pH 7.9, 150 mM, NaCl, 0.1 mM EDTA, 10% glycerol, 0.5% Tween  
436 20 lysis buffer supplemented with EDTA-free Roche protease inhibitors (reference  
437 11873580001, Merck KGaA, Darmstadt, Germany). Cell lysates were briefly sonicated and  
438 total proteins of cell extracts were quantified using Bio-Rad protein reagent assay (reference  
439 5000001, Bio-Rad Laboratories, Marnes-la-CoquetteFrance). For detection of mitochondrial  
440 proteins, mitochondria were isolated from HepaRG cells after 16h of treatment with pesticides.  
441 Cells were lysed in H-medium (HEPES 5mM, D-mannitol 210mM, sucrose 70mM, pH 7.4).  
442 The lysate was centrifuged 5 minutes at 600g, 4°C. The supernatant (S1) was centrifuged 10  
443 minutes at 4300g, 4°C. The supernatant (S2) was centrifuged 2h at 20,000g at 4°C. Resulting  
444 supernatant corresponded to cytosolic fraction. The pellet (C2) was then resuspended and  
445 centrifuged 5 minutes at 600g, 4°C. The supernatant collected (S3) was centrifuged 10 minutes  
446 at 4300g at 4°C. The pellet containing purified mitochondria was resuspended in a minimum  
447 volume of H-medium and frozen at -80°C.

448 Thirty µg of cytosolic or 10µg of mitochondrial proteins from each sample were separated  
449 on NuPAGE® Novex® Bis-Tris 4–12% gels kit (Invitrogen, Thermo Fisher Scientific, Waltham,  
450 MA, USA) and transferred to PVDF membranes (Trans-blot® Turbo™ Transfer System, Bio-  
451 Rad Laboratories, Marnes-la-CoquetteFrance) prior to immunoblotting using the following  
452 primary antibodies: COX4 (F-8, sc-376731), GSTA1 (R-14, sc-100546), eIF2a (FL-315, sc-

453 11386), anti-phospho Ser52-eIF2a (sc-12412) and HSC70 (B-6, sc-526-G) obtained from Santa  
454 Cruz Technologies (Dallas TX USA), Anti-Cytochrome C (556433, BD bioscience, San Jose,  
455 CA, USA), IRE1a (14C10, 3294 Cell Signaling Technology®, Danvers, MA, USA), anti  
456 phospho S724-IRE1a (reference ab124945, Abcam, Cambridge, United Kingdom),. Primary  
457 antibodies were detected using Dako secondary antibodies coupled to horseradish peroxidase  
458 (Agilent Technologies, Santa Clara, CA, USA), and detection of the immune complex was  
459 performed by chemiluminescent detection using Pierce™ ECL western blotting substrate  
460 (reference 32106, Thermo Fisher Scientific, Waltham, MA, USA).

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### 463 *2.10. Data analysis.*

464 All data are expressed as mean  $\pm$  standard error of the mean (SEM) from at least three  
465 independent experiments. Comparisons between groups were performed using one-way  
466 ANOVA followed by a post hoc Dunnett's test or Bonferroni's test when the normality test was  
467 positive, and Kruskal-Wallis followed by a post hoc Dunn's test when data were not normally  
468 distributed. Graph Pad Prism 8 software (GraphPad Software, San Diego CA, USA) was used  
469 for all statistical analyses) and the threshold for statistical significance was set to  $p < 0.05$ .

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### 486 3. Results

#### 487 3.1. Chronic and acute exposures to the pesticide mixture induce cytotoxicity in human 488 hepatocytes.

489 In order to study the effects of the mixture of the 7 pesticides on hepatic cell fate, we performed  
490 chronic exposures of human progenitor HepaRG cells at %ADI, ADI and 3ADI pesticide  
491 concentrations with repeated treatments at each medium renewal during 30 days (**Figure 1A**).

492 During the first two weeks, the pesticides at the ADI and 3ADI concentrations induced a slight  
493 but statistically significant increase in ATP contents (**Figure 1B**) without any visible effect on  
494 the cell morphology (**Supporting Information 3A-B**) suggesting that the phytosanitary  
495 compounds could stimulate the proliferation of progenitor HepaRG cells. In contrast, when the  
496 chronic treatment was prolonged for 2 more weeks during differentiation, the pesticides  
497 triggered a strong decrease in the ATP content in a concentration dependent manner at days  
498 21 and 30 (**Figure 1B**). Differentiated HepaRG control cultures were characterized by well-  
499 defined colonies of cuboidal hepatocytes exhibiting a dark cytoplasm, a large nucleus with a  
500 single nucleolus, and bile neo-canaliculi (**Figure 1C**), surrounded by polygonal cholangiocyte-  
501 like cells to form a confluent monolayer of differentiated HepaRG cells. At day 30 in cultures  
502 exposed to the pesticide mixture, hepatocyte-like cell colonies lost their typical morphology  
503 and we observed refringent cells and areas where cells had detached (**Figure 1C**). The alterations  
504 in cell morphology were also observed at day 21 and with the different pesticide concentrations  
505 (**Supporting Information 3A**). These data demonstrated that the pesticide mixture triggered a  
506 dose- and time-dependent cytotoxicity in differentiated HepaRG cells.

507 In order to determine whether the cytotoxicity of the pesticides observed after 2 weeks resulted  
508 from the chronic exposure of HepaRG cells during proliferation or occurred only when cells  
509 underwent differentiation, we performed acute exposures of differentiated HepaRG cells  
510 (**Figure 2**). The cells were first differentiated during 30 days in absence of pesticides, then  
511 subjected to a single exposure to the pesticide mixture (**Figure 2A**). After 2 days, we observed  
512 a strong decrease in the ATP content of differentiated HepaRG cells in a dose-dependent  
513 manner (**Figure 2B**). Significant effects were found with concentrations as low as  $\frac{1}{2}$  ADI but  
514 the overall reduction in cell viability reached 50% of the control value for the ADI and higher  
515 concentrations. These data demonstrated that the pesticide mixture also induced a strong  
516 cytotoxicity in differentiated HepaRG cells after a single acute exposure.

517 We next investigated whether the pesticides affected differently the viability of both  
518 hepatocyte- and cholangiocyte-like HepaRG cells by preparing enriched populations of both  
519 cell types incubated in the same conditions (**Figure 2A**). Interestingly, the pesticides triggered  
520 a strong cytotoxicity in the hepatocyte-like cell population while a mild decrease in ATP content  
521 was observed in the cholangiocyte cultures (**Figure 2B**). The selective cytotoxicity towards  
522 hepatocytes was also visible with the morphological alterations in hepatocyte-like cell colonies  
523 while cholangiocytes remained unaffected (**Supporting Information 3B**).

524 To further confirm the toxicity of the pesticide cocktail toward metabolically competent hepatic  
525 cells, we used pure cultures of PHH incubated in the same conditions (**Figure 2B**). The ATP  
526 content decreased in a dose-dependent manner with an extended loss in cell viability at 3 and  
527 6ADI concentrations of pesticides. These data demonstrated the high cytotoxic effect of the  
528 pesticide cocktail towards differentiated human hepatocytes. This conclusion was further  
529 supported by the absence of cytotoxicity in progenitor HepaRG cells, human HepG2 (**Figure**  
530 **2B**) and HuH7 hepatoma cells as well as primary rat liver epithelial SDVI cells, human  
531 macrophages and mesenchymal cells (**Supporting Information 3C-D**). In addition, we also  
532 used transgenic HepaRG cell lines expressing the green fluorescent protein (GFP) only in  
533 hepatocyte-like cells (**Supporting Information 3E**). Upon exposure to the pesticide mixture,  
534 the GFP positive (GFP+) hepatocyte-like cell colonies were strongly reduced in size and  
535 fluorescence intensities while cholangiocyte-like cells remained viable. Moreover, in GFP+  
536 HepaRG cells exposed to pesticides, the mRNA levels of the CYP3A4 and CYP2E1, two  
537 specific functions expressed in hepatocytes, were dramatically decreased compared to those  
538 found in untreated cells. In contrast, the cytokeratin 19 (CK19) mRNA expressed in  
539 cholangiocytes remained stable (**Supporting Information 3F**). Together, our data  
540 demonstrated that the pesticide cocktail triggered cytotoxicity specifically in hepatocytes.

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### 543 *3.2. Maneb and mancozeb induce oxidative stress and apoptosis in human hepatocytes.*

544 In order to determine whether one or several pesticides within the mixture were responsible for  
545 the toxicity, we exposed hepatocyte-like HepaRG cells to each pesticide individually in a wide  
546 range of concentrations (from ADI to 50ADI) for 48h and evaluated cell viability by measuring  
547 the ATP content (**Figure 3A**). Exposures to each pesticide have unveiled that only maneb and  
548 mancozeb were toxic. Moreover, the decreases in ATP content were similar with one or both

549 DTCs and the mixture of all compounds (**Figure 2B**) demonstrating the absence of additive  
550 effects of maneb and mancozeb and strongly suggesting that the toxicity observed with the  
551 mixture was triggered by these DTCs. To confirm this hypothesis, we exposed hepatocyte-like  
552 HepaRG cells to 7 different mixtures depleted in each pesticide individually and a mixture of 5  
553 pesticides without maneb and mancozeb (**Figure 3B**). All mixtures depleted in one compound  
554 induced the same decrease in ATP contents while the cocktail of 5 pesticides without the two  
555 DTCs had no effect on cell viability confirming that maneb and mancozeb induced the toxicity  
556 of the mixture and evidencing for the first time that these DTCs trigger cell death in human  
557 hepatocytes at very low concentrations in the range of the ADI.

558 We next studied the mechanism of cytotoxicity induced by the pesticide mixture and DTCs  
559 (**Figure 4**) postulating that maneb and mancozeb may induce apoptosis. We measured caspase  
560 3, 8 and 9 catalytic activities in total cell lysates of hepatocyte-like HepaRG cells exposed to  
561 the pesticide mixture or mancozeb alone (**Figure 4A**). The initiator caspase 9 and executioner  
562 caspase 3 activities were significantly increased in cells incubated with the mixture and  
563 mancozeb while the caspase 8 activity was unchanged suggesting the lack of cell death receptor  
564 activation. Importantly, when the antioxidant NAC was added prior to the pesticide mixture,  
565 the increase in caspase 3 and 9 activities was totally abrogated. Similarly, the pesticide mixture  
566 strongly induced the caspase 3 activities in human hepatocytes in primary culture (**Figure 4B**).  
567 The induction of apoptosis was further demonstrated by the cytochrome *c* release from  
568 mitochondria in both differentiated HepaRG cells and human hepatocytes (**Figure 4C**) and the  
569 increase in subG1 fraction of hepatocyte-like HepaRG cells (**Supporting Information 4A**).  
570 Together, these data demonstrated that the pesticide mixture and mancozeb induced caspase-  
571 dependent apoptosis in human hepatocytes through an intrinsic pathway and suggested the  
572 involvement of oxidative stress since NAC prevented the onset of cell death.

573 The effects of pesticides on mitochondrial function were next assessed in differentiated  
574 HepaRG cells. While basal respiration was not affected, maximal respiration and respiration  
575 linked to ATP production were significantly decreased upon exposure to the pesticide mixture  
576 at the 3ADI concentration (**Figure 4D**). In contrast, proton leak was increased by the same  
577 concentration. The activities of the five OXPHOS mitochondrial complexes were also studied  
578 in control culture and in cells exposed to maneb and no significant differences in enzymatic  
579 activities could be observed between untreated and pesticide-exposed cells (**Supporting**

580 **information 4B**), thus suggesting an impairment of other metabolic pathways linked to  
581 OXPPOS.

582 The oxidative stress was next evaluated by spectrofluorometry using the CellROX, H<sub>2</sub>DCFDA  
583 and MitoSOX probes. While the CellROX indicator evidenced oxidative stress only at high  
584 concentrations of pesticides, H<sub>2</sub>DCFDA and MitoSOX probes indicated that relative oxidative  
585 stress levels were strongly enhanced in cells exposed to the pesticide mixture or mancozeb  
586 alone at ADI and 3ADI concentrations (**Figure 4E**). Importantly, NAC totally prevented  
587 pesticide-induced oxidative stress measured with H<sub>2</sub>DCFDA and MitoSOX probes and the  
588 cytotoxicity evaluated by the ATP content. In addition, no significant change in the relative  
589 ROS levels were observed in cholangiocyte-like HepaRG cells treated with the same  
590 concentrations of pesticides (**Supporting Information 4C**). Oxidative stress was further  
591 investigated in hepatocyte-like HepaRG cells (**Figure 4F**) and human hepatocytes (**Figure 4G**)  
592 by measuring the mRNA levels of genes known to be regulated by ROS and/or involved in  
593 antioxidant responses. Superoxide dismutase 1 (SOD1) mRNA levels were decreased while  
594 those of SOD2, glutathione peroxidase 3 (GPX3), BCL2-associated X apoptosis regulator  
595 (BAX) and DNA damage inducible transcript 3 (DDIT3, also known as CHOP) were up-  
596 regulated upon exposure to the pesticide mixture. The increase in DDIT3 transcripts suggested  
597 the induction of an endoplasmic reticulum (ER) stress, which was confirmed with the up-  
598 regulation of PERK and ERN1 mRNA levels and the increase in IRE1 $\alpha$  phosphorylation and  
599 eIF1 $\alpha$  protein expression (**Supporting Information 4D**). Considering the evidence of ROS  
600 overproduction and ER stress response in hepatocyte-like HepaRG cells incubated with the  
601 pesticides, a genotoxic effect was investigated using the  $\gamma$ -H2AX assay (**Supporting**  
602 **information 4E-F**). A strong induction in the percentage of  $\gamma$ -H2AX positive cells was found  
603 in cells exposed to the pesticide mixture and aflatoxin B1 used as a positive control of  
604 genotoxicity. Altogether, these results indicated that the pesticide mixture and DTCs triggered  
605 a burst of ROS inducing ER stress, mitochondrial dysfunction, genotoxicity and apoptosis  
606 through an intrinsic caspase pathway.

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609 *3.3. Mancozeb is metabolized in hepatocytes to produce ethylene thiourea.*

610 Since cell death induced by DTCs occurred only in metabolically competent hepatocyte-like  
611 HepaRG cells and human hepatocytes but not in cholangiocyte-like and progenitor HepaRG  
612 cells, we compared the catabolism of the mancozeb in these different cell cultures.

613 Detection of mancozeb was assessed by HPLC coupled to UV detector (HPLC-UV) (**Figure 5**)  
614 and mancozeb's metabolites were identified by LC-MS/MS (**Table 2, Supporting**  
615 **Information 2**). Mancozeb added to culture media at the concentration of 100  $\mu$ M was detected  
616 by HPLC-UV at 285nm with a retention time of 10.75 min (**Figure 5**). This peak was confirmed  
617 to be mancozeb by LC-MS/MS (**Table 2, Supporting Information 2**). In addition, in media  
618 supplemented with mancozeb, a second peak was detected by HPLC at 247nm at a retention  
619 time of 2.7 min, very similar retention time to that of ethylene thiourea (ETU) analytical  
620 standard (**Figure 5**). Using LC-MS/MS (**Table 2**), we confirmed the presence of  $\sim$ 21  $\mu$ M of  
621 ETU in culture media without incubation with HepaRG cells strongly suggesting the presence  
622 of this degradation product in the mancozeb stock solution. In culture media of hepatocyte-like  
623 HepaRG cells, the amounts of mancozeb progressively decreased during a 24h incubation time  
624 while those of ETU concomitantly increased (**Figure 5, Table 2**). In contrast, the peaks of  
625 mancozeb and ETU detected by HPLC in culture media of cholangiocyte-like and progenitor  
626 HepaRG cells were not quantitatively modified during the 24h incubation with these cells  
627 (**Figure 5**). These data demonstrated that mancozeb is catabolized into its main ETU metabolite  
628 in hepatocyte-like HepaRG cells and that progenitor and cholangiocyte-like cells do not  
629 metabolize mancozeb.

630 In addition to the peaks of mancozeb and ETU, we also detected by HPLC-UV two other  
631 compounds eluting with retention times of 3.1 and 4.5 min only in culture media of hepatocyte-  
632 like HepaRG cells (**Figure 5A**). The peak at 3.1 min was not detected after 3h of incubation  
633 with mancozeb in hepatocyte-like HepaRG cells. However, the area of this peak increased with  
634 the incubation times and was similar in both untreated and treated cultures at 24h demonstrating  
635 that it corresponded to an endogenous hepatocyte-like HepaRG metabolite unrelated to  
636 mancozeb metabolism. This hypothesis was further confirmed by the absence of this peak in  
637 media of cholangiocyte-like HepaRG cells (**Figure 5B**) cultured in the same medium than  
638 hepatocyte-like HepaRG cells.

639 In a detailed LC-MS/MS analytical study, we investigated the presence of other known  
640 metabolites previously reported (Engst and Schnaak, 1970 ; World Health Organization 1988 ;  
641 IRNS 2010), such as ethylene urea and ethylene diamine (**Supporting Information 2**) in the



642 culture media of hepatocyte-like HepaRG cells in order to identify the compound eluting at 4.5  
643 min in HPLC chromatograms (**Supporting Information 4**). We found that all known  
644 mancozeb metabolites were either undetectable or present at concentrations below the  
645 quantification limits (**Supporting Information 2**). By LC-MS/MS analysis, we found that the  
646 unknown compound eluting at 4.5 min in HPLC-UV had a molecular mass of 152 g/mol. Using  
647 the MASS BANK database, we identified two putative candidates, xanthine and oxipurinol,  
648 two compounds of the purine metabolic pathway. The LC-MS/MS analysis of commercial  
649 standards of these two compounds demonstrated that the unknown compound was xanthine  
650 (**Supporting Information 2, Table 2**). Although xanthine was detected in the culture media of  
651 control cells unexposed to mancozeb ( $\sim 2.7 \mu\text{M}$  at 24h of culture), its levels were significantly  
652 increased up to  $10.4 \mu\text{M}$  in media of hepatocyte-like HepaRG cells incubated with  $100 \mu\text{M}$   
653 mancozeb (**Table 2**). We next analyzed hypoxanthine and uric acid, two other main compounds  
654 of the purine metabolic pathway. We found that hypoxanthine was also produced by  
655 hepatocyte-like HepaRG cells incubated with mancozeb but not uric acid. Quantification of the  
656 lactate dehydrogenase (LDH) activities showed no significant differences between culture  
657 media of cells exposed or not to mancozeb (**Table 2**) indicating that the increase in  
658 hypoxanthine and xanthine levels upon mancozeb treatment was due to an active secretion  
659 rather than a passive leakage from cells resulting from loss of plasma membrane integrity in  
660 mancozeb-treated cells. Since hypoxanthine and xanthine have not been identified as  
661 metabolites of DTCs, we hypothesized that their efflux was a stress response resulting from  
662 mancozeb exposure.

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### 665 *3.4. Exposure to DTCs leads to Mn overload and Zn depletion and apoptosis in hepatocytes.*

666 In order to determine if the production of DTC metabolites by hepatocyte-like HepaRG cells  
667 was responsible for DTC cytotoxicity, we exposed these cells to ETU as well as ethylene urea  
668 (EU) and ethylene diamine (EDA) and the combination of the three metabolites at  
669 concentrations ranging from 2 to  $20 \mu\text{M}$  corresponding to mancozeb at ADI to  $10\times\text{ADI}$   
670 concentrations (**Figure 6A**). Exposure to these molecules had no significant effects on cell  
671 viability demonstrating that these metabolites were not involved in the cytotoxicity of DTCs  
672 towards hepatocytes.

673 Several DTCs, including maneb, zineb and mancozeb, are salts containing Mn and/or Zn. While  
674 maneb and zineb contain only Mn and Zn, respectively, mancozeb is a combination of both

675 maneb and zineb (**Supporting Information 2**). We hypothesized that catabolism of DTCs in  
676 hepatocytes could lead to the release of Mn and/or Zn, present in their chemical structure. To  
677 address this hypothesis, hepatocyte-like HepaRG cells were exposed for 48h to maneb,  
678 mancozeb and zineb at the 3ADI concentration, as well as  $\text{MnCl}_2$  and  $\text{ZnCl}_2$  at  $7\mu\text{M}$   
679 corresponding to the concentration of Mn and Zn brought by exposure to DTCs at 3ADI  
680 (**Figure 6B**).

681 Quantification of the relative ATP contents in hepatocyte-like HepaRG cells demonstrated that  
682 exposure to maneb, mancozeb and  $\text{MnCl}_2$  triggered similar toxicities while zineb and  $\text{ZnCl}_2$  had  
683 no significant impact of cell viability (**Figure 6B**). The similar experiment performed using  
684 cholangiocyte-like cells showed no effect on cell viability with any of these treatments. These  
685 data strongly suggested that cell death of hepatocyte-like HepaRG cells exposed to maneb and  
686 mancozeb resulted from the release of Mn during their catabolism. We next studied the effects  
687 of exposures to maneb and  $\text{MnCl}_2$  on the intracellular contents in Mn and Zn measured by  
688 ICP/MS in progenitor, cholangiocyte- and hepatocyte-like HepaRG cells (**Figure 6C-D**,  
689 **Supporting Information 5**). Hepatocytes exposed to maneb and  $\text{MnCl}_2$  showed strong  
690 intracellular and mitochondrial accumulations of Mn with contents significantly higher than in  
691 untreated cells. Intracellular amounts of Mn were also increased in cholangiocytes and  
692 progenitor cells but in much lower extents compared those of hepatocyte-like cells. Importantly,  
693 significant decreases in intracellular Zn levels were also observed upon incubations with  $\text{MnCl}_2$   
694 and maneb only in hepatocyte-like cells. Together, these data supported the conclusion that Mn-  
695 containing DTCs induced cell death in metabolically competent hepatocytes capable of  
696 metabolizing these fungicides to produce innocuous metabolites, such as ETU, and to release  
697 Mn accumulating in cells and altering Zn content.

698 Since the treatment of hepatocyte-like HepaRG cells by  $\text{MnCl}_2$  induced cytotoxicity, we used  
699 this experimental condition to determine whether the production of hypoxanthine and xanthine  
700 previously observed with mancozeb occurred in  $\text{MnCl}_2$ -treated cells (**Figure 5, Table 2**). We  
701 found that exposure of differentiated HepaRG cells to  $\text{MnCl}_2$  also enhanced the secretion of  
702 both xanthine and hypoxanthine (**Table 2**) demonstrating that these compounds are not  
703 metabolites resulting from DTC catabolism and suggesting that they may be produced in  
704 response to the alteration of the intracellular Mn/Zn content and/or oxidative stress.

705 To further study the role of the Zn depletion in DTC- and Mn-dependent toxicity, hepatocyte-  
706 like HepaRG cells were exposed to the pesticide mixture, maneb, mancozeb or  $\text{MnCl}_2$  in  
707 absence or presence of a 10-fold excess of  $\text{ZnCl}_2$  ( $70\mu\text{M}$ ) and oxidative stress, relative ATP  
708 content, caspase 3 and 9 activities and intracellular Mn and Zn amounts were measured (**Figure**  
709 **7**). First, incubation of differentiated HepaRG cells with  $\text{MnCl}_2$  led to an increase in general  
710 oxidative stress measured with  $\text{H}_2\text{DCFDA}$  probe and relative mitochondrial superoxide levels  
711 measured with MitoSOX probe at similar levels to those observed in cultures exposed to maneb  
712 or mancozeb while fluorescence measured with the CellROX probe was increased with  
713 pesticide mixture and mancozeb only (**Figure 7A**). In addition, co-treatment with  $70\mu\text{M}$   $\text{ZnCl}_2$   
714 prevented the oxidative stress measured with  $\text{H}_2\text{DCFDA}$  and MitoSOX probes (**Figure 7A**),  
715 the decrease in ATP content (**Figure 7B**) and the onset of apoptosis (**Figure 7C**) in cultures  
716 exposed to DTCs and  $\text{MnCl}_2$ . Determination of cellular Mn and Zn contents in untreated control  
717 cells and cultures exposed to  $\text{MnCl}_2$  and maneb in presence of  $70\mu\text{M}$  of  $\text{ZnCl}_2$  (**Figure 7D**,  
718 **Supporting Information 5**) demonstrated that the 10-fold excess in Zn partially reduced the  
719 intracellular and mitochondrial Mn overload and significantly prevented Zn depletion induced  
720 by maneb and  $\text{MnCl}_2$ . Unexpectedly, while the excess in Zn increased the intracellular Zn  
721 content in absence of maneb and  $\text{MnCl}_2$ , it also reduced the mitochondrial contents in Zn in all  
722 culture conditions. These results demonstrated that partial restoration of Mn and Zn  
723 homeostasis disrupted by Mn-containing DTCs was sufficient to prevent oxidative stress and  
724 cytotoxicity in hepatocytes.

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#### 735 4. Discussion

736 The initial goal of this work was to study the effects of a mixture of 7 pesticides (chlorpyrifos-  
737 ethyl, dimethoate, diazinon, iprodione, imazalil, maneb, mancozeb) often detected in food samples  
738 (EFSA, 2017) at low concentrations in human hepatocytes *in vitro*. We evidenced that the pesticide  
739 mixture induced a strong cytotoxicity in hepatocytes-like HepaRG cells and human hepatocytes  
740 during chronic and acute exposures. The pesticides triggered the production of ROS, an ER stress  
741 response and DNA damages, which led to cell death by intrinsic apoptosis involving caspases 3  
742 and 9. Unexpectedly, the individual evaluation of each pesticide demonstrated that the toxicity  
743 was carried by the Mn-containing DTCs maneb and mancozeb as the mixture of the five other  
744 pesticides without these compounds did not affect viability, and that the exposure to maneb or  
745 mancozeb alone recapitulated the effect of the mixture.

746 DTCs are broad-spectrum fungicides reacting with amino acids and enzymes. They are used on  
747 many fruits, vegetables, nuts, and field crops and spread on potatoes, corn, sorghum, tomatoes,  
748 apples, pears, grapes, onions and cereal grains to protect against fungal diseases. DTCs have been  
749 employed for decades with the examples of maneb and mancozeb registered on the market since  
750 1948 and 1961, respectively. Nearly 200,000 tons of maneb are spread every year in the world and  
751 the overall amounts of mancozeb is estimated to several millions (Gullino et al., 2010 ; National  
752 Toxicology Program, 2014). Maneb was banned in 2017 in E.U. and but it is still used worldwide  
753 in large amounts. In 2018, the Agence Nationale de Sécurité Sanitaire de l'Alimentation, de  
754 l'Environnement et du Travail (ANSES) recommended to the European Commission the non-  
755 renewal of the mancozeb approval given risks for human health and considering the high levels of  
756 exposure resulting of its intensive use (ANSES, 2018). EU has adopted the non-renewal of  
757 mancozeb (Official Journal of the European Union, 2020), which was withdrawn in 2021 with a  
758 grace period that expired in January 2022.

759 Despite available data regarding DTC toxicity *in vivo* and *in vitro*, there are still ongoing  
760 investigations to decipher their mechanisms of action. Maneb and mancozeb show low acute  
761 toxicity *in vivo* (median lethal dose/LD<sub>50</sub> >1g/kg), but adverse effects have been described in  
762 rodents upon chronic exposure (5 to 10 mg/kg b.wt./d) with neurotoxicity and hyperthyroidism  
763 (World Health Organization 1988 ; IRNS, 2010). Chronic occupational or experimental exposures  
764 to maneb induce Parkinson-like syndromes in humans and animals (Ferraz et al., 1988 ;  
765 Thiruchelvam et al., 2002 ; Anderson et al., 2021). Their main metabolite ETU also shows low  
766 toxicity (median lethal dose/LD<sub>50</sub> >1.5g/kg b.wt.) in rodents. However, ETU is a structural analog

767 of thionamides, which inhibits the thyroid peroxidase, reduces the synthesis of triiodothyronine  
768 ( $T_3$ ) and thyroxin ( $T_4$ ) and induces thyroid hypertrophy (Panganiban et al., 2004 ; Goldner et al.,  
769 2010), defining an endocrine disrupting effect. ETU also alters the proliferation/survival of murine  
770 hematopoietic cell lineages with the development of pancytopenia in long-term exposures (Porreca  
771 et al., 2016). In addition, reports also suggest that occupational exposure to maneb and mancozeb  
772 induces alterations in lymphocyte and monocyte blood counts (Corsini et al., 2005 ; Colosio et al.,  
773 2007).

774 The *in vivo* hepatotoxicity of DTCs is less described with, however, studies demonstrating that  
775 mancozeb at high doses, 313.6 mg/kg b.wt./d, 3 times a week for 6 weeks (Sakr, 2007) and 750  
776 mg/kg b.wt./d for 10 weeks (Hashem et al., 2018) induce liver damages with disruption of the  
777 normal histology, necrosis and mononuclear cell infiltration, depletion in glutathione, lipid  
778 peroxidation, genotoxicity and induction of inflammation in rats. Interestingly, antioxidant  
779 molecules such as *Nigella sativa* oil (Hashem et al., 2018) and curcumin (Saber et al., 2019) reduce  
780 the hepatotoxicity induced by mancozeb. Demonstration has also been brought that ETU (125  
781 mg/kg, single dose) induces hepatic morphological and histological abnormalities in newborns  
782 from female rats exposed during gestation, with strong remodeling of the hepatic trabeculae and  
783 severe hepatic megakaryocytosis (Lemos et al., 2012).

784 *In vitro* studies have described mitochondrial-mediated cytotoxicity and apoptosis of maneb and/or  
785 mancozeb in various cell types including rat mesencephalic primary embryonic cells (Domico et  
786 al., 2007), SK-N-AS neuroblastoma cells (Anderson et al., 2018), AGS gastric carcinoma cells  
787 (Kumar et al., 2019), HepG2 hepatoma cells (Lori et al., 2021) and HT-29 and Caco2 colon cells  
788 (Dhaneshwar et al., 2021, Hoffman and Hardej, 2012 ; Hoffman et al., 2016) through production  
789 of reactive oxygen species (ROS). In these reports, the cells were exposed to a wide range of  
790 concentrations of maneb and/or mancozeb from 1 to 500  $\mu$ M, and the cytotoxic effects were  
791 observed at high concentrations from 10 to 100 $\mu$ M, except for rat mesencephalic primary  
792 embryonic cells in which ROS production was detected at 3 $\mu$ M (Domico et al., 2007).

793 To the best of our knowledge, our study is the first report describing induction of oxidative stress  
794 and apoptosis by maneb and mancozeb in differentiated hepatocyte-like HepaRG cells and primary  
795 human hepatocytes, two *in vitro* models of metabolically competent hepatocytes expression XMEs  
796 (Aninat et al., 2006 ; Kanebratt and Anderson, 2008). In these cell models, the adverse effects  
797 occurred at concentrations as low as  $\frac{1}{2}$ ADI (1.13  $\mu$ M) in acute exposures and at hNDI (0.73  $\mu$ M)  
798 in chronic treatments demonstrating that human hepatocytes are particularly sensitive to maneb-  
799 and mancozeb-induced toxicity. Interestingly, cytotoxic effects at such low concentrations were

800 not found in other hepatoma cell lines and non-hepatic cells, which do not express phase I and II  
801 XMEs enabling pesticide metabolism. In this report, we showed that human hepatocytes  
802 metabolize mancozeb to produce ETU, which correlated with intracellular accumulation in Mn  
803 and depletion in Zn triggering ROS overproduction, xanthine/hypoxanthine release, ER stress and  
804 apoptosis.

805 It has been previously shown that maneb and mancozeb alter metal contents in HT-29 and Caco2  
806 colon cells (Hoffman and Hardej, 2012 ; Hoffman et al., 2016). In these studies, the authors  
807 demonstrated that maneb and mancozeb cause Mn overload but also strongly enhanced Zn and  
808 copper (Cu) cellular contents (Hoffman and Hardej, 2012). Nevertheless, the same authors also  
809 reported that DTC namab, which contains sodium but not Mn, affected Cu and Zn contents in a  
810 much lower extent than maneb and mancozeb supporting the involvement of Mn in oxidative stress  
811 induced by maneb and mancozeb (Hoffman and Hardej, 2012). In another recent study, authors  
812 reported that rats exposed to mancozeb (at 100 mg/kg b. wt. via oral gavage once daily for 28 days)  
813 showed no alteration of hepatic histology but a slight increase in Mn and glutathione contents, and  
814 higher glutathione peroxidase and reductase activities in the liver (Kistingner and Hardej, 2022).  
815 However, in the same study, the use of namab (at 95mg/kg b. wt.) recapitulated the effects of  
816 mancozeb on the liver suggesting that the organic backbone of DTCs but not the associated-metal  
817 (Mn or Zn) was mainly responsible for the alteration of hepatic redox status (Kistingner and Hardej,  
818 2022).

819 In this context of contradictory data, our study further supports the mechanism of toxicity of the  
820 Mn-containing DTCs maneb and mancozeb through their biotransformation in hepatocytes leading  
821 to intracellular Mn accumulation and depletion in zinc (Zn), which are the key events in the  
822 observed cytotoxicity. The conclusion that alteration of the Mn/Zn homeostasis plays crucial role  
823 in hepatocyte death is based on the following observations 1) exposure to ETU did not induce  
824 hepatocyte cell death, 2) toxicity of maneb was prevented by a 10-fold excess in ZnCl<sub>2</sub>, which  
825 partially restored the Mn/Zn contents indicating that Zn/Mn intracellular flux are predominant in  
826 the pesticide-mediated toxicity, 3) the DTC zineb, with chemical structure similar to maneb and  
827 mancozeb but containing only Zn, did not induce hepatocyte cytotoxicity, and 4) exposure of  
828 hepatocytes to MnCl<sub>2</sub> produced similar adverse effects than maneb and mancozeb.

829 While Mn is an essential metal because of its role as co-factor of multiple enzyme-mediated  
830 catalytic activities, high Mn overload resulting from occupational overexposure causes the well-  
831 described manganism, a neurological syndrome similar to Parkinson's disease (Kim et al., 2015).  
832 Even in non-occupationally exposure (i.e. lower doses of exposure), Mn remains one of the most

833 toxic metals with endocrine disrupting effects especially during pregnancy with impacts on birth  
834 outcomes (Tsai et al., 2015 ; Signes-Pastor et al., 2019). Through its ability to cross the blood-  
835 brain barrier more efficiently in infants because of an incompletely developed blood–brain barrier  
836 and their inability to fully eliminate metals, Mn accumulates in the hypothalamus to affect the  
837 timing of puberty (Dees et al., 2017).

838 The characterization of Mn homeostasis was recently established with the identification of the  
839 membrane transporters DMT1 (*SLC11A2*), ZIP18 (*SLC39A8*), *SLC30A10* and ZIP14 (*SLC39A14*)  
840 regulating Mn uptake and efflux in enterocytes, cholangiocytes and hepatocytes (Katz and Rader,  
841 2019). Importantly, these transporters also regulate transport of other metals including Zn, which  
842 is consistent with our data showing that excess in ZnCl<sub>2</sub> abolished the deleterious effects of DTCs  
843 and MnCl<sub>2</sub> in hepatocytes. This observation may be of interest in order to prevent adverse effects  
844 of Mn-containing DTCs during occupational exposure through a specific diet containing Zn  
845 supplementation.

846 The principal risks of exposure to DTCs are expected to be inhalation resulting from spray drift  
847 from application sites (van Wendel de Jood et al., 2014) and residues present on agriculture  
848 products (EFSA, 2017). Despite the fact that most DTCs will soon be banned in E.U., chronic  
849 exposure of worldwide populations to maneb and manozeb at low doses is likely to persist in the  
850 future and the impact of both ETU and Mn as endocrine disruptors needs to be considered  
851 especially for individuals with liver diseases. In healthy individuals, excess of Mn in blood or liver  
852 is released into the bile and eliminated with the stool (Katz and Rader, 2019). Patients suffering  
853 from liver diseases could be more prone to Mn toxicity because of the impairment of bile  
854 production and/or Mn excretion. Consistently, patients with liver cirrhosis have increased blood  
855 and brain Mn contents that may contribute to the occurrence of neurodegenerative disorders  
856 (Mehkari et al., 2020). In this context and in the light of our data, the impact of long-term exposures  
857 to low doses of Mn-containing DTCs especially in the liver remains to be investigated.

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## 860 **5. Conclusion**

861 This work provides new insight into the toxicity of manganese Mn-containing DTCs maneb and  
862 mancozeb in metabolically competent human hepatocytes. Very low doses of maneb and  
863 mancozeb induce oxidative stress triggering caspase-dependent apoptosis. The mechanism of  
864 cytotoxicity of Mn-containing DTCs involves their biotransformation in hepatocytes and the  
865 release of Mn leading to intracellular Mn overload and depletion in zinc (Zn). Alteration of these

866 metal's homeostasis provokes oxidative stress and apoptosis, which both can be prevented by Zn  
867 supplementation.

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882 repositories will be publicly available at Mendeley Data website if the manuscript is accepted.

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### 885 **Conflict of Interest**

886 The authors declare that they have no conflict of interest regarding this work.

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889 **Author Contributions:** Conceptualization, P.L.O., A.C. and B.F.; methodology, K.P., S.B.,  
890 C.R., M.R., O.L., L.A., N.B. and P.L.O.; validation, L.A., N.B. and P.L.; formal analysis, P.L.O.,  
891 B.F. and A.C.; investigation, K.P., Y.V., S.B., C.R., C.A., P.LE.; resources, L.A., N.B. and  
892 M.R.; data curation, A.C. and P.L.O.; writing—original draft preparation, P.L.O.; writing—  
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895 authors have read and agreed to the published version of the manuscript.

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**899 Data Sharing**

900 Information about data sharing protocols, options for accessing data, and links to data  
901 repositories may be provided in the “Acknowledgments” section, as noted above. Authors may  
902 also provide links to data repositories in the “Methods” or “Results” sections of their  
903 manuscripts, as appropriate.

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## 1232 **Figure legends**

1233

1234 **Table 1 :** CAS numbers, molecular weights (MW in g/mol), and values of ADI (mg/kg.bw/day,  
1235 from EFSA 2010) and the highest Nutritional Daily Intake (hNDI) or Theoretical Maximum  
1236 Daily Intake (TMDI), presented as %ADI ( $\mu\text{g}/\text{kg.bw}/\text{day}$ ) for each pesticide. These values were  
1237 converted to mg/L and  $\mu\text{M}$  for *in vitro* studies. The ratio (%) of each individual pesticide was  
1238 calculated from their concentration in  $\mu\text{M}$  within the %ADI and ADI mixtures.

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1240

1241 **Table 2:** Quantification of mancozeb, ethylene thiourea, hypoxanthine, xanthine and uric acid  
1242 by LC-MS/MS (expressed in  $\mu\text{M} \pm\text{SD}$ ) and lactate dehydrogenase enzymatic activity(LDH,  
1243 expressed in mU/mL) in culture media of hepatocyte-like HepaRG cells after 3, 8 and 24h of  
1244 incubation with mancozeb at 100  $\mu\text{M}$ . Statistics:  $p < 0.05$  *a* significantly different from 3h, *b*  
1245 significantly different from 8h, \* significantly different from untreated control (DMSO), #  
1246 significantly different from Mz 100 $\mu\text{M}$ .

1247

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## 1249 **Figure 1**

1250 **Chronic exposure of HepaRG cells to the pesticide mixture at low concentrations. A.**

1251 Schematic representation of the differentiation procedure of HepaRG cells over 30 days of  
1252 culture and protocol of chronic exposure to the pesticide mixture at different concentrations  
1253 (%ADI, ADI or 3ADI). Photographs showing the changes in density and cell morphologies at  
1254 days 1, 7, 14, 21 and 30 days after plating in untreated control cell cultures. One day after cell  
1255 seeding at low density, proliferating progenitor cells were treated (day 1), then every 2 or 3  
1256 days at each renewal of culture medium (3 times a week) during the process of differentiation.  
1257 Cell viability assays (ATP content) were performed at days 7, 14, 21 and 30, after 3, 6, 9 and

1258 12 treatments, respectively. **B.** Cell viability evaluated by measuring the intracellular ATP  
1259 content after 7, 14, 21 and 30 days of exposure in progenitor and differentiated HepaRG cells  
1260 exposed to repeated treatments. Data were expressed as percentages of untreated control cells.  
1261 Results are means  $\pm$  SEM ( $N \geq 3$ ), \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from control (Ctrl)  
1262 using One-way ANOVA and Dunnett's multiple comparisons test. **C.** Micrographs in phase  
1263 contrast of control and pesticide-treated (3ADI) cultures at day 30. White arrows indicate  
1264 hepatocyte- (Hep) and cholangiocyte-like cells. Bars: 100  $\mu$ m.

1265

1266

## 1267 **Figure 2**

### 1268 **Acute exposure of hepatic cells to the pesticide mixture at low concentrations. A.**

1269 Schematic representation of the protocol of acute exposure of differentiated HepaRG cells to  
1270 the pesticide mixture at different concentrations (%ADI, 1/4, 1/2, 1 and 3ADI). Progenitor cells  
1271 were plated and differentiated during 30 days in absence of pesticides. Upper panel:  
1272 Differentiated cells were exposed to a single treatment for 48h. Photographs showing the  
1273 changes in cell morphologies between control and treated cultures. Lower panel: Differentiated  
1274 hepatocyte- and cholangiocyte-like cells were selectively detached and seeded separately to  
1275 obtain enriched cultures of both cell phenotypes. Two days after plating, hepatocyte- and  
1276 cholangiocyte-like cells were exposed to a single treatment for 48h. Photographs showing the  
1277 changes in cell morphologies between control and treated cultures.

1278 **B.** Cell viabilities of differentiated HepaRG cells, human hepatocytes in primary culture and  
1279 HepG2 hepatoma cells were measured by quantification of the intracellular ATP content after  
1280 48h of exposure at different concentrations of the pesticide mixture (1/4ADI, 1/2ADI, ADI,  
1281 3ADI, 6ADI or %ADI). Data were expressed as % of untreated control cells. Results are means  
1282  $\pm$  SEM ( $N \geq 3$ ), \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  significantly different from control (Ctrl)  
1283 using One-way ANOVA and Dunnett's multiple comparisons test.

1284

1285

## 1286 **Figure 3**

### 1287 **Hepatotoxicity of the pesticide mixture is triggered by maneb and mancozeb. A.**

1288 Cell viability was evaluated by measuring intracellular ATP contents in hepatocyte-like HepaRG  
1289 cells either in untreated control cultures (Ctrl) or exposed for 48h to each pesticide individually  
1290 and the coincubation with maneb and mancozeb at different concentrations (%ADI, ADI, 3ADI,

1291 10ADI, 25ADI, 50ADI). **B.** Cell viability (ATP content) in hepatocyte-like HepaRG cells  
1292 incubated for 48 h to 7 pesticide mixtures depleted in each pesticide (6 compounds per mixture)  
1293 and a cocktail of 5 pesticides without maneb and mancozeb at different concentrations (%ADI,  
1294 1/4, 1/2, 1, 3 and 6ADI). Data were expressed as % of control. Results are means  $\pm$  SEM ( $N \geq 3$ ),  
1295 \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from control (Ctrl) using One-way ANOVA +  
1296 Dunnett's or Kruskal Wallis + Dunn's multiple comparisons tests.

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1299 **Figure 4.**

1300 **Pesticide mixture and mancozeb induce oxidative stress and apoptosis in human**  
1301 **hepatocytes.** **A.** Caspase 8, 9 and 3 activities were quantified using Ac-IETD-AMC, Ac-  
1302 LEHD-AMC and Ac-DEVD-AMC fluorogenic substrates, respectively, in hepatocyte-like  
1303 HepaRG cells exposed for 24h to the pesticide mixture at different concentrations (%ADI, ADI  
1304 and 3ADI), in absence or presence of N-acetylcysteine (NAC, 20 mM), and mancozeb (Mz) at  
1305 the 3ADI concentration. **B.** Ac-DEVD-AMC substrate caspase 3 activity quantified in human  
1306 hepatocytes in primary culture exposed to the pesticide mixture for 48h at different  
1307 concentrations (%ADI, ADI and 3ADI). Relative data were normalized to the basal values  
1308 measured in untreated control cells arbitrarily set as 1 (Ctrl). Results are means  $\pm$  SEM ( $N \geq 3$ ),  
1309 \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  significantly different from control (Ctrl). **C.** Detection of  
1310 cytochrome *c* by western blot in cytosol and mitochondrial subcellular fractions of  
1311 differentiated HepaRG cells and human hepatocytes in untreated control (Ctrl) conditions and  
1312 exposed to the pesticide mixture for 24h at the ADI and 3ADI concentrations. **D.** Parameters of  
1313 mitochondrial respiration (basal and maximal respiration, ATP production and proton leak)  
1314 were analyzed in differentiated HepaRG cells exposed for 16h to the pesticide mixture (%ADI,  
1315 ADI and 3ADI concentrations) using XF Cell Seahorse Mito Stress Test [Results are means  $\pm$   
1316 SEM ( $N \geq 3$ ), One-way ANOVA + Bonferroni's multiple comparisons test or Kruskal Wallis +  
1317 Dunn's multiple comparisons test]. **E.** Oxidative stress was evaluated in hepatocyte-like  
1318 HepaRG cells using CellROX, H<sub>2</sub>DCFDA and MitoSOX probes after 24h exposure to pesticide  
1319 mixture (%ADI, ADI or 3ADI) or mancozeb alone (Mz 3ADI). N-acetyl cysteine (NAC 20mM)  
1320 was used to neutralize oxidative stress (Kruskal Wallis + Dunn's multiple comparisons test).  
1321 Cell viability of hepatocyte-like HepaRG cells was quantified by the relative intracellular ATP  
1322 content after 24h exposure to pesticide mixture (%ADI, ADI or 3ADI) or mancozeb alone (Mz  
1323 3ADI). **F-G.** Relative mRNA levels of *SOD1* and 2, *GPX3*, *BAX* and *DDIT3* genes in

1324 hepatocyte-like HepaRG cells (**F**) and human hepatocytes (**G**) exposed for 24h to the pesticide  
1325 mixture (%ADI, ADI or 3ADI). Data were expressed as % of the untreated control culture  
1326 (Ctrl). Results are means  $\pm$  SEM ( $N \geq 3$ ), \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , significantly  
1327 different from control (Ctrl).

1328

1329

### 1330 **Figure 5**

1331 **Hepatocyte-like HepaRG cells metabolize mancozeb.** Representative UV-HPLC  
1332 chromatograms of culture media without mancozeb (untreated control cells at 24h, Ctrl) or  
1333 containing mancozeb (100 $\mu$ M) and incubated for 3, 8 and 24h with **A**) hepatocyte-like, **B**)  
1334 cholangiocyte-like and **C**) progenitor HepaRG cells. Mancozeb, ethylenethiourea (ETU) and  
1335 xanthine peaks are indicated in panel A.

1336

1337

### 1338 **Figure 6**

1339 **Exposure to maneb induces Mn overload and Zn depletion triggering cytotoxicity in**  
1340 **hepatocytes.** **A.** Effects of DTCs metabolites ethylene thiourea (ETU), ethylene urea (EU) and  
1341 ethylene diamine (EDA) metabolites and the combination of the three molecules  
1342 (EDA+ETU+EU) at 2, 10 and 20  $\mu$ M on cell viability (relative ATP contents) in hepatocyte-  
1343 like HepaRG cell after 48h of exposure. **B.** Cell viability (relative ATP contents) in hepatocyte-  
1344 and cholangiocyte-like HepaRG cell cultures after 48h treatments with the pesticide mixture  
1345 (mixture), DTCs maneb (Mb), mancozeb (Mz), zineb (Zb) at 3ADI concentration,  $MnCl_2$  and  
1346  $ZnCl_2$  at 7 $\mu$ M. Relative ATP contents are expressed as % of untreated control cultures (Ctrl).  
1347 Results are means  $\pm$  SEM ( $N \geq 3$ ), \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from control (Ctrl)  
1348 with Kruskal Wallis + Dunn's multiple comparisons test. **C.** Relative intracellular (**C**) and  
1349 mitochondrial (**D**) Mn and Zn contents in progenitor, hepatocyte- and cholangiocyte-like  
1350 HepaRG cells measured by ICP/MS after 16h of exposures to maneb (3ADI) and  $MnCl_2$  at  
1351 7 $\mu$ M. Data are expressed as fold change in metal contents between unexposed control cells  
1352 arbitrary set as 1 (dotted line) and treated cells because of the large differences in metal contents  
1353 between cell types and intracellular versus mitochondrial amounts. Absolute quantities of both  
1354 Mn and Zn are presented in ng/well in the Supporting Information 5. Results are mean  $\pm$  SEM  
1355 ( $N \geq 3$ ), \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from control (Ctrl) One-way ANOVA +  
1356 Dunnett's multiple comparisons test or Kruskal-Wallis + Dunn's multiple test

1357 **Figure 7**1358 **Excess of Zn prevents oxidative stress and cytotoxicity induced by DTCs in hepatocytes.**

1359 **A.** Relative oxidative stress levels evaluated in hepatocyte-like HepaRG cells using CellROX,  
1360 H<sub>2</sub>DCFDA and MitoSOX probes and, **B.** relative ATP contents in hepatocyte-like HepaRG  
1361 cells after 24h of exposure to the pesticide mixture (3ADI), DTCs maneb (Mb 3ADI) and  
1362 mancozeb (Mz 3ADI), and MnCl<sub>2</sub> at 7μM in absence or presence of a 10-fold-excess in ZnCl<sub>2</sub>  
1363 at 70μM. For oxidative stress, statistics are: One-way ANOVA + Sidak's multiple comparisons  
1364 test or Kruskal-Wallis + Dunn's multiple comparisons test, \*p< 0.05, \*\*p< 0.01, significantly  
1365 different from untreated control cultures (Ctrl), #p< 0.05, significantly different from cultures  
1366 exposed for 24h to the pesticide mixture, maneb, mancozeb and MnCl<sub>2</sub> in absence of a 10-fold-  
1367 excess in ZnCl<sub>2</sub> at 70μM. For ATP contents, results are means ± SEM (N≥3), statistics are:  
1368 Kruskal-Wallis + Dunn's multiple comparisons test, \*p< 0.05, \*\*p< 0.01, significantly different  
1369 from untreated control (Ctrl). **C.** Relative caspase 3 and 9 activities quantified using Ac-DEVD-  
1370 AMC and Ac-LEHD-AMC fluorogenic substrates, respectively, after exposure to the pesticide  
1371 mixture (3ADI) and mancozeb (Mz 3ADI) in absence or presence of a 10-fold-excess in ZnCl<sub>2</sub>  
1372 at 70μM. Kruskal-Wallis + Dunn's multiple comparisons test. Results are means ± SEM (N≥3),  
1373 \*p< 0.05, \*\*p< 0.01, \*\*\*p< 0.001, significantly different from untreated control cultures (Ctrl)  
1374 and from cells co-exposed to excess of Zn. **D-E.** Relative intracellular (**D**) and mitochondria  
1375 (**E**) Mn and Zn contents measured by ICP/MS after 16h exposures of hepatocytes HepaRG to  
1376 maneb (3ADI) and MnCl<sub>2</sub> at 7μM in absence (-) and presence (+) of a 10-fold excess of ZnCl<sub>2</sub>  
1377 (70μM). Data are expressed as fold change of untreated control cells (Ctrl). Results are means  
1378 ± SEM (N≥3).

1379

**Table 1**

Pesticides	CAS Number	MW (g/mol)	hNDI/TMDI	%ADI or hNDI			ADI	ADI		
			in %ADI	Concentrations and percentages			EFSA 2010	Concentrations and percentages		
				$\mu\text{g/L}$	$\mu\text{M}$	% ( $\mu\text{M}$ ) of each pesticide in mixture	mg/kg/day	mg/L	$\mu\text{M}$	% ( $\mu\text{M}$ ) of each pesticide in mixture
Chlorpyrifos	2921-88-2	350,586	3.3	3.51	0.01	0.63	0.01	0.12	0.3423	4.22
Diazinon	333-41-5	304,346	36.67	0.88	0.00286	0.18	0.0002	0.0024	0.0078	0.096
Dimethoate	60-51-5	229,257	24.67	2.29	0.01	0.63	0.001	0.012	0.0523	0.645
Imazalil	35554-44-0	297,18	6.87	20.8	0.07	4.43	0.025	0.3	1.009	12.44
Iprodione	36734619-7	330,167	0.47	3.38	0.01	0.63	0.06	0.72	2.181	26.9
Maneb	12427-38-2	265,302	32.07	192.42	0.73	46.2	0.05	0.6	2.262	27.9
Mancozeb	8018-01-7	271,3	32.07	192.42	0.72	45.57	0.05	0.6	2.251	27.77
Mixture				424.58	1.58	100		2.3544	8.1054	100



Table 2:

	Hours	Mancozeb	Ethylene thiourea	Hypoxanthine	Xanthine	Uric acid	LDH (mU/mL)
<b>DMSO</b>	3	<0.05	<0.1	1.70 ± 0.40	1.42± 0.30	0.64 ± 0.42	2.15 ± 1.68
	8	<0.05	<0.1	0.48 ± 0.10	2.08± 0.82	2.21± 2.35	3.92 ± 0.89
	24	<0.05	<0.1	0.75 ± 0.35	2.69 ± 3.36	10.34 ± 8.21	8.19 ± 3.66
<b>Mancozeb (100µM)</b>	3	118.28 ± 6.82	21.40 ± 0.65	0.62 ± 0.22	0.75 ± 0.16	0.32 ± 0.16	2.19 ± 0.86
	8	104.79 ± 4.52 <sup>a</sup>	20.73 ± 0.71	1.28 ± 0.93 <sup>a</sup>	2.29± 0.69 <sup>a</sup>	2.48 ± 1.98	4.10 ± 0.71
	24	35.44 ± 2.91 <sup>a,b</sup>	64.58 ± 2.58 <sup>a,b</sup>	5.15 ± 3.29 <sup>a</sup>	10.36 ± 3.63 <sup>a</sup>	9.47 ± 5.49	10.10 ± 1.96
<b>MnCl<sub>2</sub> (100µM)</b>	3	<0.05	<0.1	0.99 ± 0.45	1.00 ± 0.18	0.27 ± 0.14	1.97 ± 0.61
	8	<0.05	<0.1	2.97 ± 2.78	3.92 ± 1.08	1.59 ± 1.18	4.46 ± 0.94
	24	<0.05	<0.1	6.96 ± 3.39 <sup>a,*</sup>	13.66 ± 0.11 <sup>a,*</sup>	9.22 ± 1.71	14.37 ± 1.96 <sup>*</sup>

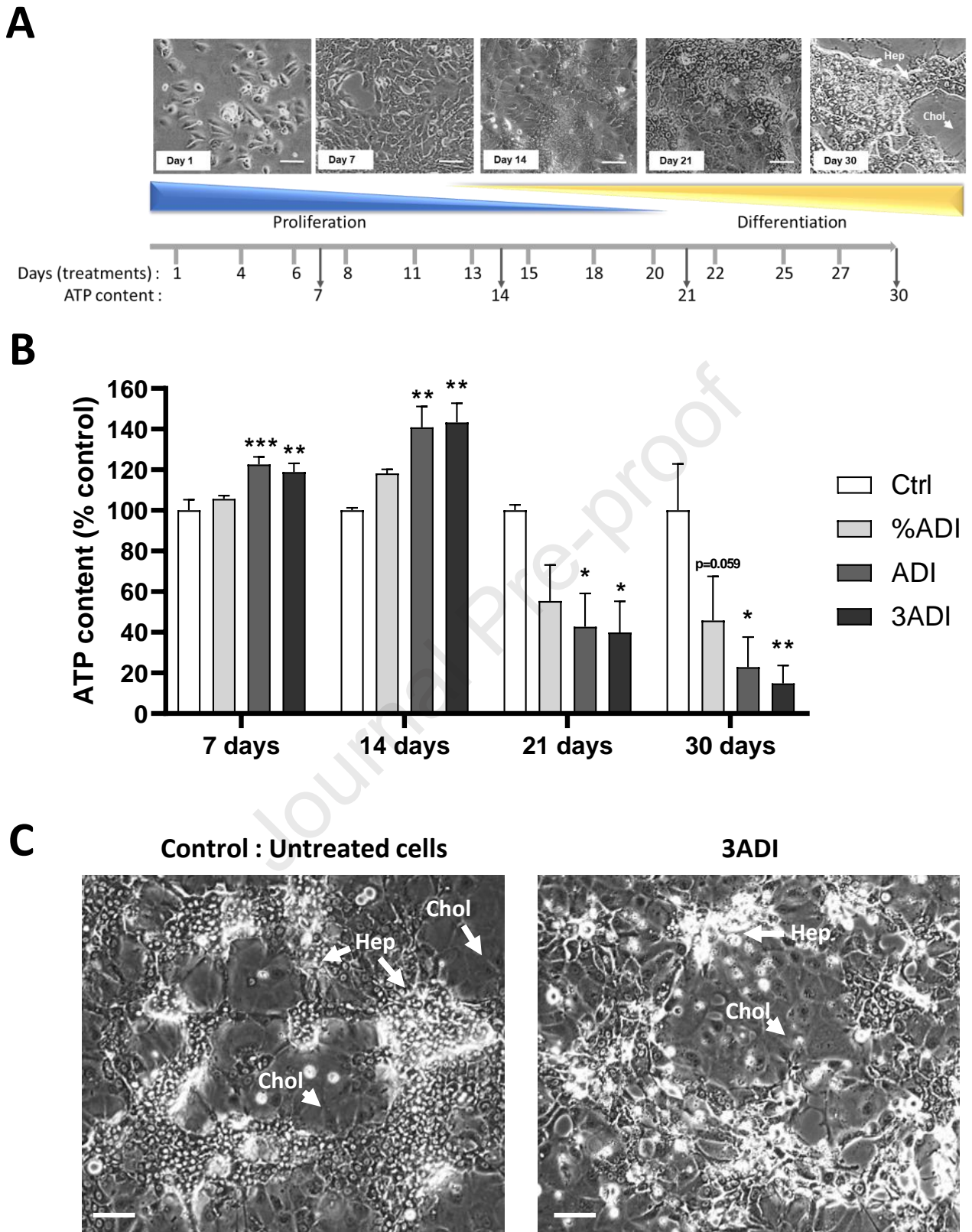


Figure 1

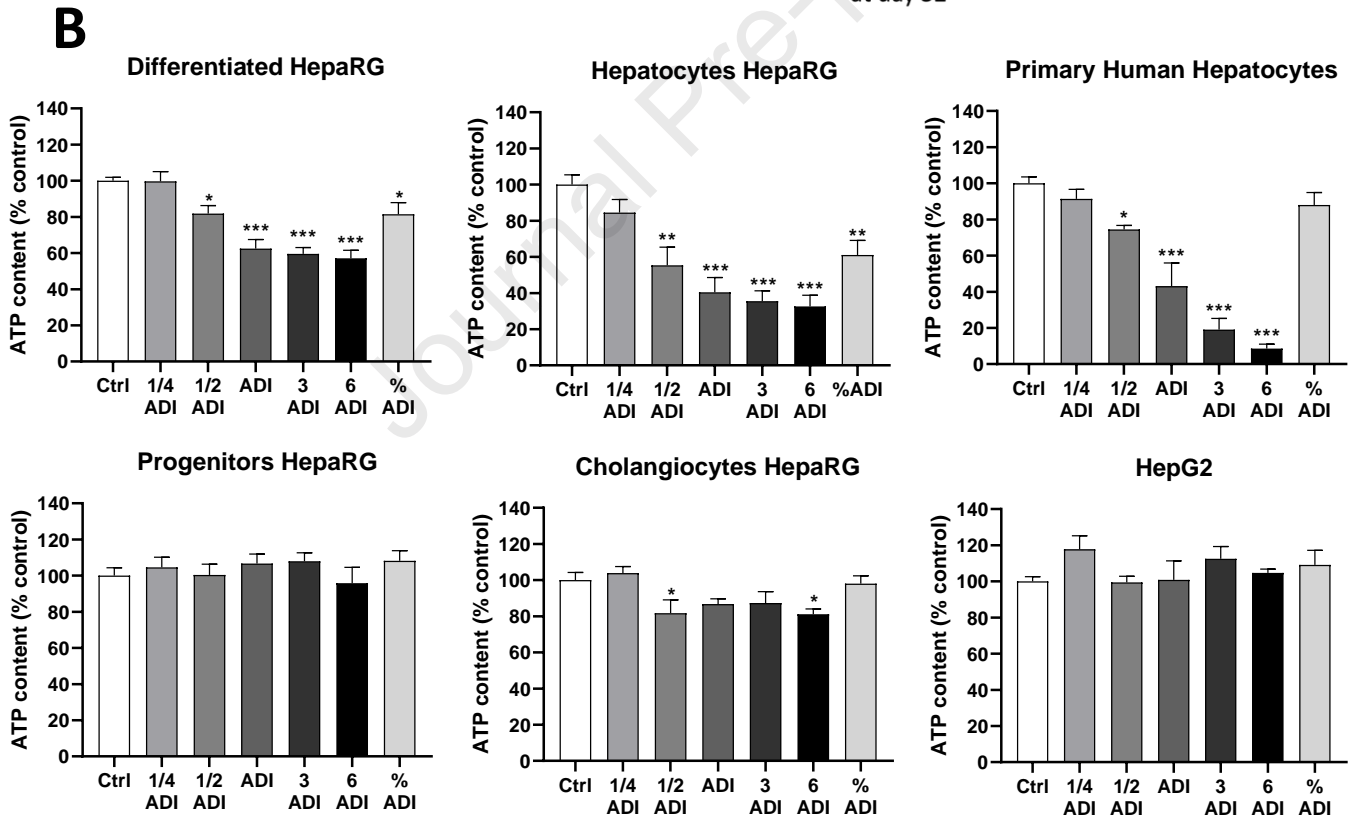
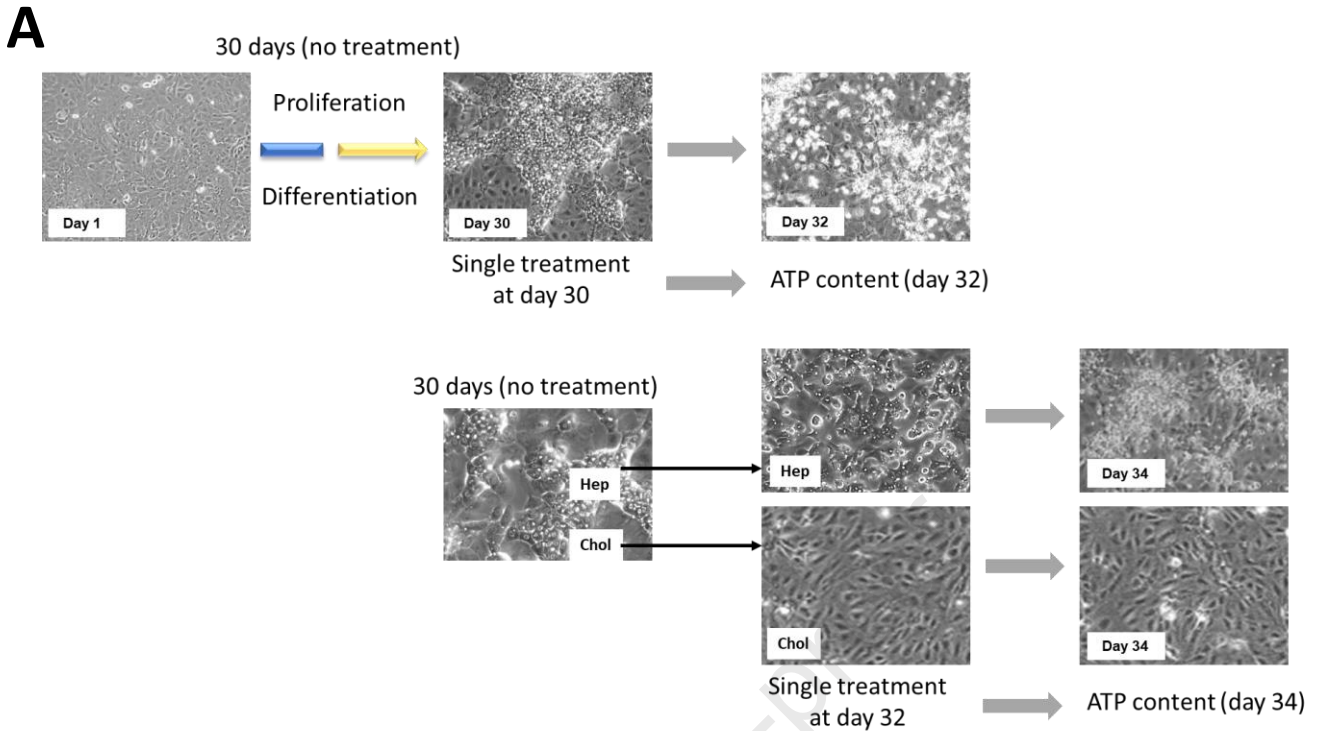
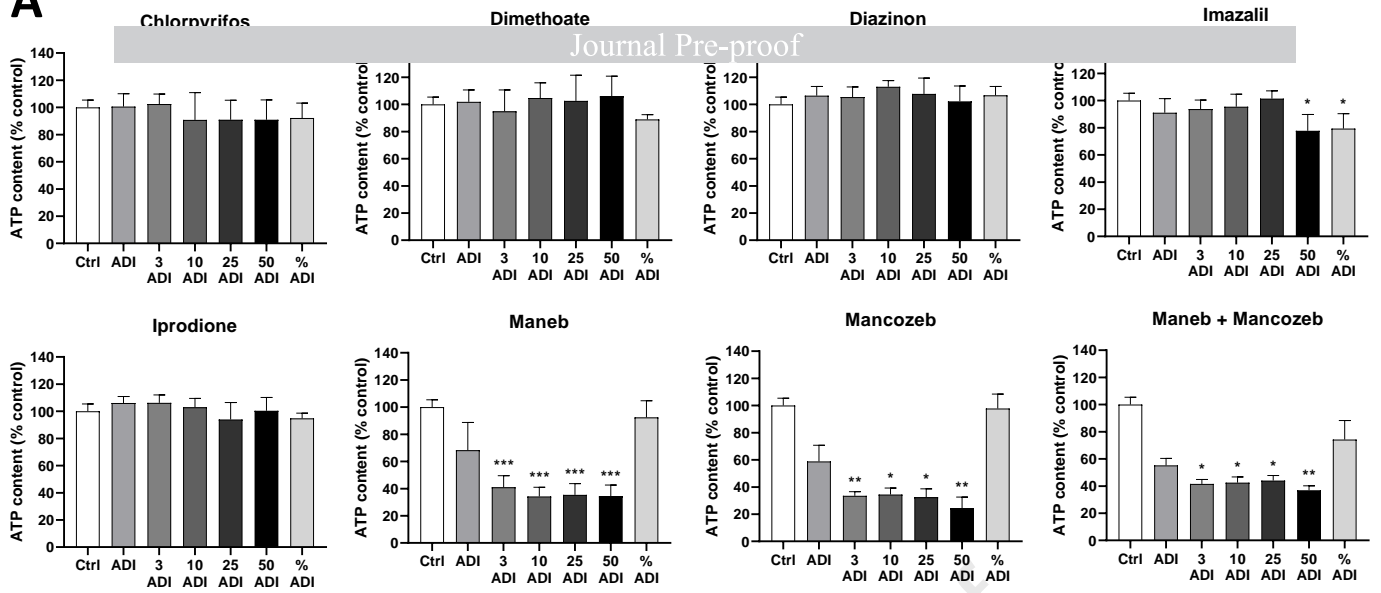
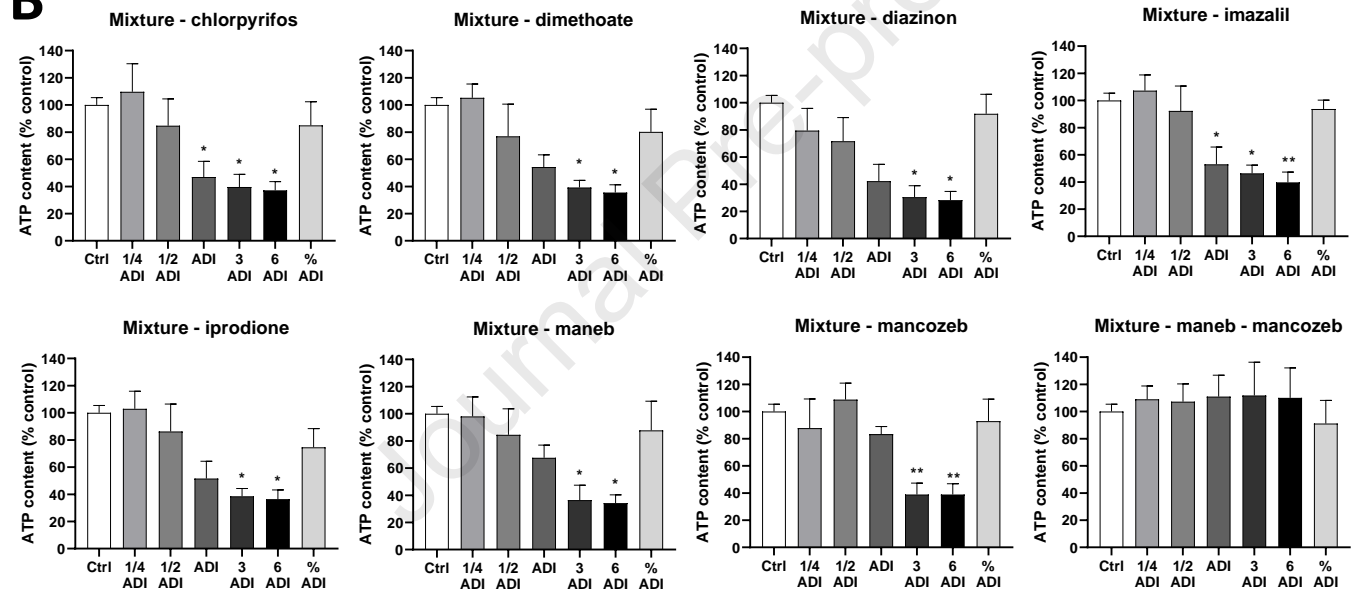
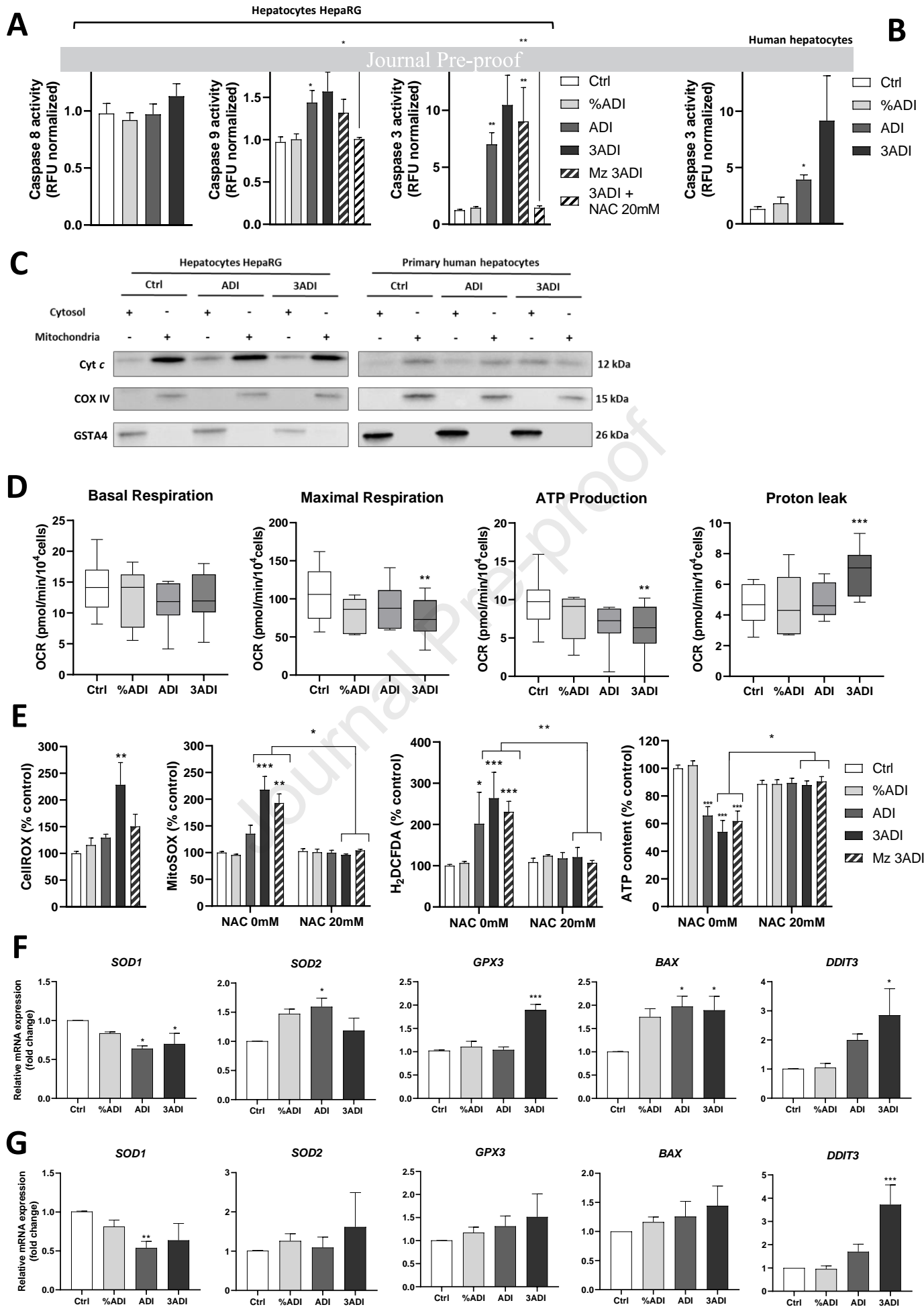


Figure 2

**A****B****Figure 3**



**Figure 4**

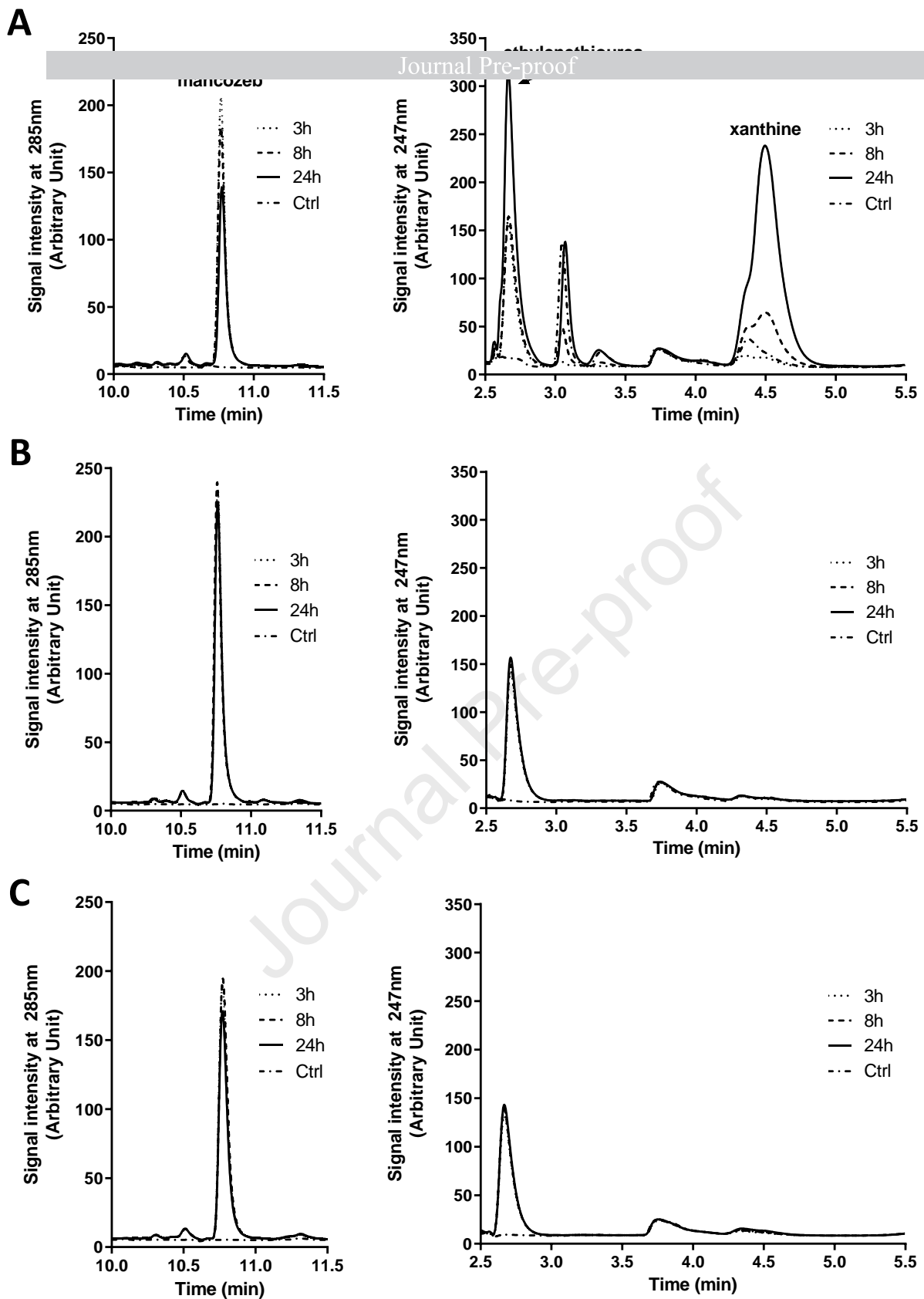


Figure 5

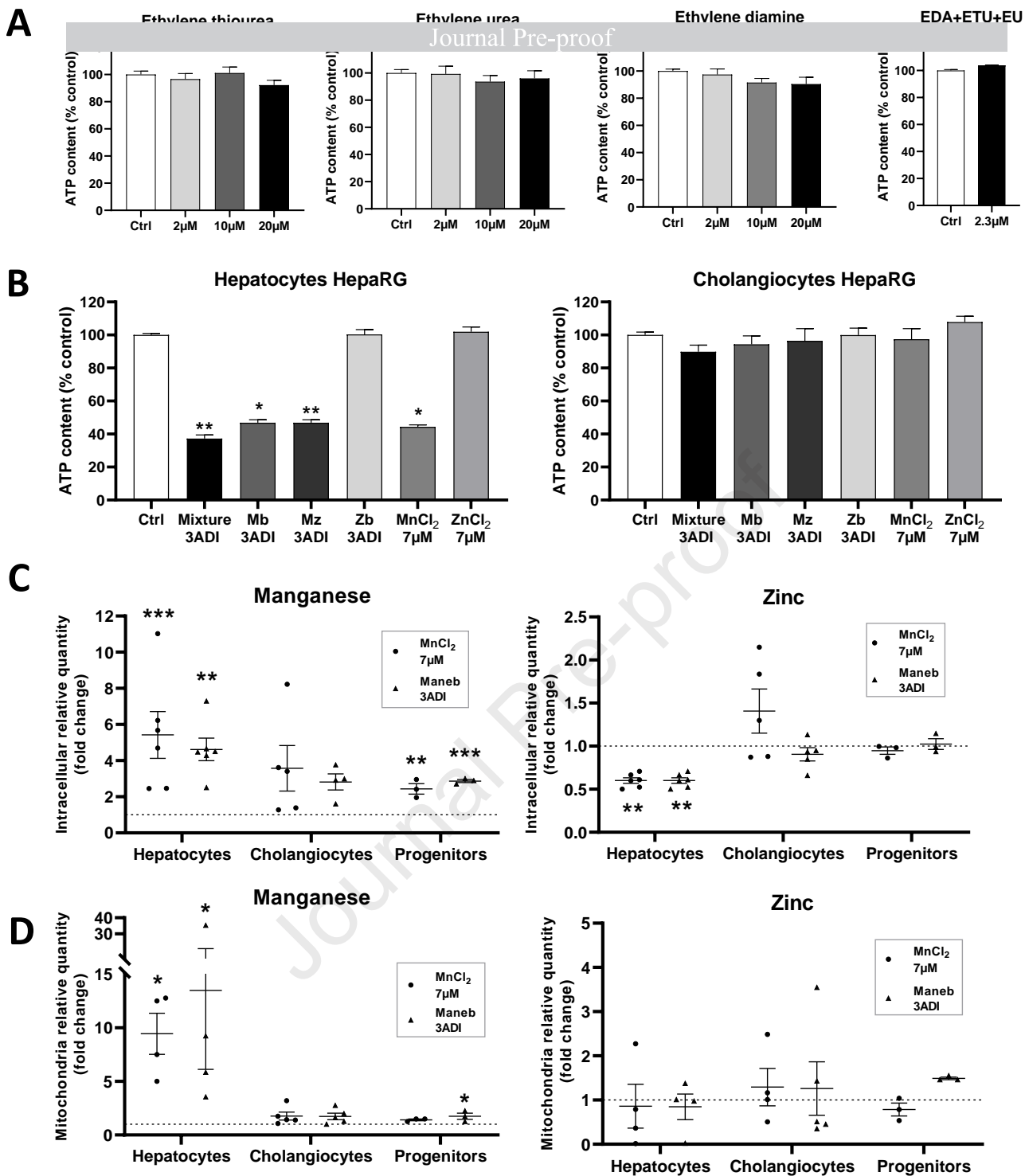
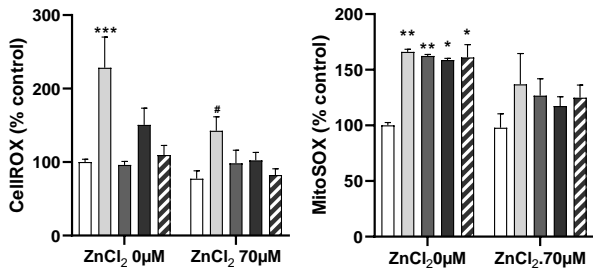
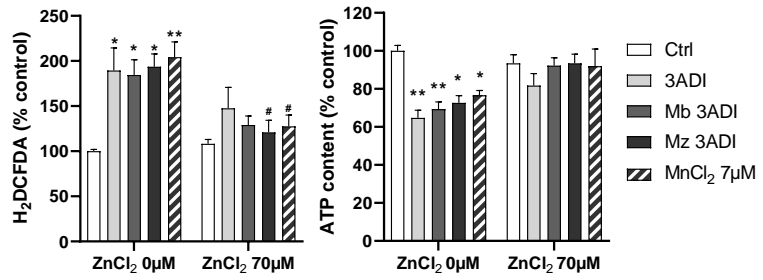
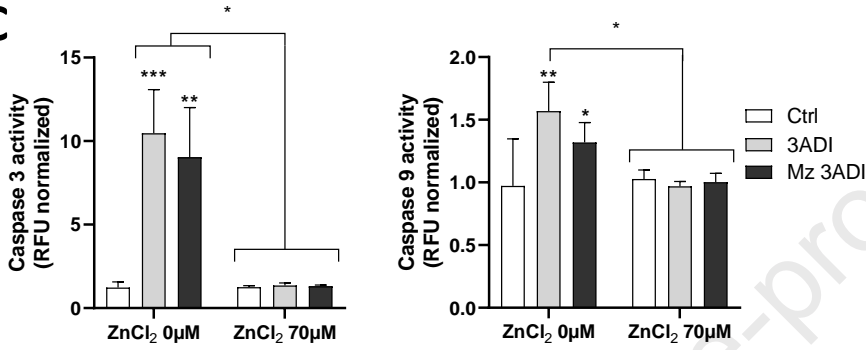
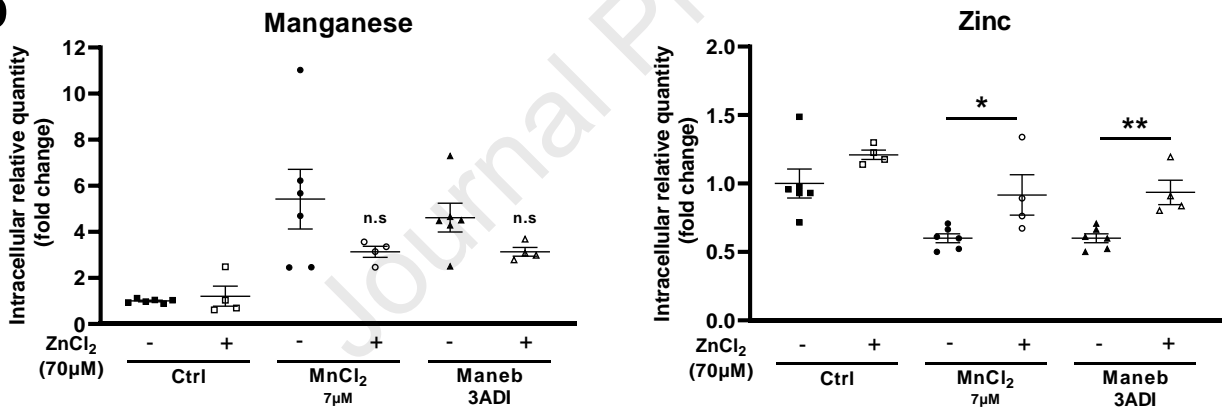
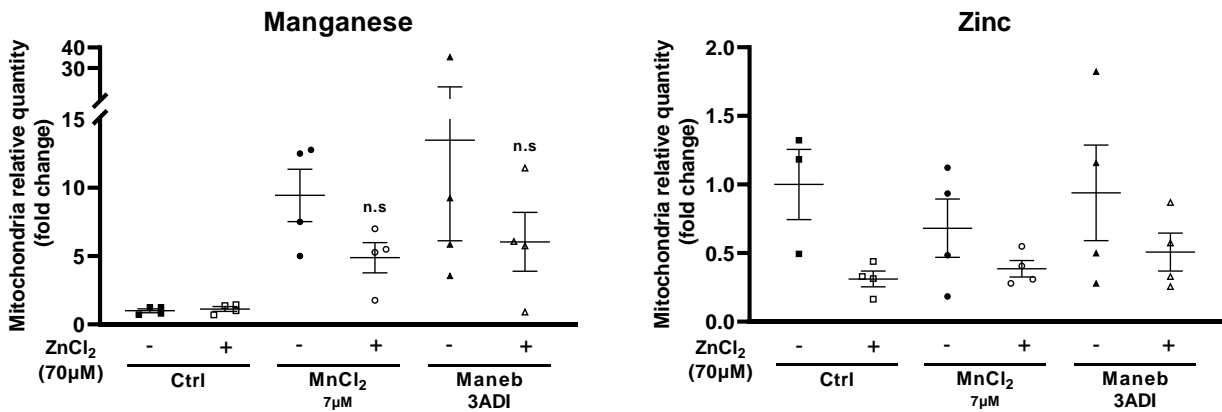


Figure 6

**A****B****C****D****E****Figure 7**



Petitjean et al “Low concentrations of ethylene bisdithiocarbamate pesticides maneb and mancozeb impair manganese and zinc homeostasis to induce oxidative stress and caspase-dependent apoptosis in human hepatocytes”.

Bullet points :

- Pesticides maneb and mancozeb induce cell death in human hepatocytes
- Induction of oxidative stress leads to caspase-dependent apoptosis
- Toxicity involves pesticide metabolization, manganese overload and zinc depletion
- Zinc supplementation prevents oxidative stress and apoptosis

### 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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