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► To cite this version:

Anne Platel, Romain Dusautoir, Gwenola Kervoaze, Gonzague Dourdin, Eulalie Gateau, et al.. Comparison of the in vivo genotoxicity of electronic and conventional cigarettes aerosols after subacute, subchronic and chronic exposures. *Journal of Hazardous Materials*, 2022, *Journal of Hazardous Materials*, 423, pp.127246. <10.1016/j.jhazmat.2021.127246>. <hal-03895890>

HAL Id: hal-03895890

<https://lilloa.hal.science/hal-03895890v1>

Submitted on 16 Oct 2023

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1 **Comparison of the *in vivo* genotoxicity of electronic and conventional cigarettes**
2 **aerosols after subacute, subchronic and chronic exposures.**

3

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61 **ABSTRACT**

62 Tobacco smoking is classified as a human carcinogen. A wide variety of new products, in particular
63 electronic cigarettes (e-cigs), have recently appeared on the market as an alternative to smoking.
64 Although the *in vitro* toxicity of e-cigs is relatively well known, there is currently a lack of data on their long-
65 term health effects. In this context, the aim of our study was to compare, on a mouse model and using a
66 nose-only exposure system, the *in vivo* genotoxic and mutagenic potential of e-cig aerosols tested at two
67 power settings (18W and 30W) and conventional cigarette (3R4F) smoke. The standard comet assay,
68 micronucleus test and *Pig-a* gene mutation assay were performed after subacute (4 days), subchronic (3
69 months) and chronic (6 months) exposure. The generation of oxidative stress was also assessed by
70 measuring the 8-hydroxy-2'-deoxyguanosine and by using the hOGG1-modified comet assay. Our results
71 show that only the high-power e-cig and the 3R4F cigarette induced oxidative DNA damage in the lung
72 and the liver of exposed mice. In return, no significant increase in chromosomal aberrations or gene
73 mutations were noted whatever the type of product. This study demonstrates that e-cigs, at high-power
74 setting, should be considered, contrary to popular belief, as hazardous products in terms of genotoxicity in
75 mouse model.

76 **KEYWORDS**

77 E-cigs

78 *In vivo* comet assay

79 *In vivo* micronucleus test

80 *In vivo* *Pig-a* gene mutation assay

81 Oxidative DNA damage

82

83

84

85 **ABBREVIATIONS**

86 e-cig: electronic cigarette; Mb18W: Modbox e-cig model set at 18 W; Mb30W: Modbox e-cig model set at
87 30 W; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; hOGG1: human 8-oxoguanine glycosylase; *Pig-a*:
88 phosphatidylinositol glycan, class A (gene); TI: tail intensity; MNPCE: micronucleated polychromatic
89 erythrocytes; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; RET: reticulocytes;
90 RBC: red blood cells; PAHs: polycyclic aromatic hydrocarbons; ALI: air-liquid interface.

91

92 1. INTRODUCTION

93 Tobacco use is a major public health concern killing more than 8 million people every year
94 worldwide, yet is the leading cause of preventable death worldwide (World Health Organization, 2020).
95 Cigarette smoke is composed of a mixture of toxicants and carcinogens such as polycyclic aromatic
96 hydrocarbons (PAHs), N-nitrosamines, aromatic amines, aldehydes (*e.g.* formaldehyde and
97 acetaldehyde), phenols, volatile hydrocarbons, and metals (International Agency for Research on Cancer,
98 2012, 2004). Tobacco smoking is classified as a human carcinogen (group 1) by the International Agency
99 for Research on Cancer (IARC) for the development of mainly lung cancer and several other cancers
100 (larynx, pharynx, oesophagus, stomach, colon, liver, pancreas, bladder, cervix, ...) (International Agency
101 for Research on Cancer, 2012, 2004). It is also well known that smoking is a major risk factor for many
102 other adverse effects on human health, including respiratory, cardiovascular, nervous, immune, liver,
103 urinary, gastrointestinal and reproductive systems (Altamirano and Bataller, 2010; Dechanet et al., 2011;
104 Erhardt, 2009; Gotts et al., 2019; Lakier, 1992; Li et al., 2014; Orth, 2000; Soares and Melo, 2008; Sopori,
105 2002; Sopori and Kozak, 1998). In recent years, a wide variety of new products, in particular electronic
106 cigarettes (e-cigs), have emerged on the market as an alternative to smoking tobacco products. E-cigs are
107 battery-powered devices that allow the nebulization of e-liquids composed of propylene glycol and/or
108 glycerol and flavoring agents, with or without nicotine. E-cigs are generally perceived as less harmful than
109 traditional cigarettes, particularly because they do not contain tobacco, do not require combustion during
110 use and deliver fewer toxicants (Cao et al., 2021; Dusautoir et al., 2021). However, due to the thermal
111 degradation of the e-liquid constituents, other substances in e-cig aerosols have been identified as toxic
112 compounds or potential carcinogens such as aldehydes (*e.g.* formaldehyde, acetaldehyde, methylglyoxal,
113 acrolein), phenolic compounds (*e.g.* phenol, quinol, catechol, ortho-, meta- and para-cresol), volatile
114 organic compounds (*e.g.* xylene, toluene, acetonitrile) and heavy metals (*e.g.* nickel and copper) (Beauval
115 et al., 2019, 2017, 2016; Cao et al., 2021; Erythropel et al., 2019; Gillman et al., 2016; Merecz-Sadowska
116 et al., 2020; Polosa et al., 2019). Moreover, due to the lack of in-depth toxicity assessment, especially long
117 term or repeated-dose toxicity studies, safety of e-cigs cannot be guaranteed.

118

119 Recent reviews (Cao et al., 2021; Merecz-Sadowska et al., 2020; Polosa et al., 2019; Wang et al.,
120 2019) provide an overview of the *in vitro* and *in vivo* toxicity of e-cigs. To date, the published studies
121 mainly focus on *in vitro* toxic effects of e-cigs emissions. Data show that exposure to e-cig aerosols

122 triggers cytotoxic effects such as cell death, DNA damage, and reactive oxygen species (ROS) and
123 proinflammatory agents' production. It was also demonstrated that e-cig aerosol is much less cytotoxic
124 than traditional cigarette smoke (Anthérieu et al., 2017; Cervellati et al., 2014; Dusautoir et al., 2021;
125 Neilson et al., 2015; Tellez et al., 2021). *In vitro*, e-cigs had the potential to increase oxidative stress and
126 inflammatory response in a similar level to that of cigarette smoke, but after more intensive exposures
127 (Dusautoir et al., 2021). In contrast, there are relatively few *in vivo* experimental approaches. Most of them
128 were performed or sponsored by the tobacco industry and have been carried out using short-term
129 exposures (from a few hours to a few weeks), with individual e-cig components (*e.g.* polyethylene glycol
130 and/or vegetable glycerine) but not whole aerosols, or with old generation or low-power e-cig devices.
131 Furthermore, the experimental protocols of exposure often used did not correspond to normal conditions
132 of use (*e.g.* number of puffs/min), or used whole body exposure systems that are not representative of real
133 exposure since animals are exposed by other routes than the respiratory route (*i.e.* cutaneous and
134 digestive by deposition of e-cig aerosols on the coat). Another limitation of most of these studies is that
135 they did not compare the results obtained with e-cig aerosol with those obtained with traditional cigarette
136 smoke. Results showed that e-cig aerosols are likely to induce oxidative stress, mitochondrial dysfunction,
137 pulmonary inflammation, DNA damage and even impairment of respiratory function (Canistro et al., 2017;
138 Garcia-Arcos et al., 2016; Glynos et al., 2018; Hwang et al., 2016; Laube et al., 2017; Lerner et al., 2016;
139 Lim and Kim, 2014; McGrath-Morrow et al., 2015; Salturk et al., 2015; Scott et al., 2018; Werley et al.,
140 2016).

141
142 Regarding the assessment of the genotoxicity of tobacco products, many studies have been
143 conducted to specifically investigate the *in vitro* genotoxic/mutagenic potential of cigarette smoke and e-
144 cig aerosols. Unfortunately, contradictory results have often been obtained depending on the products
145 tested (*e.g.* particulate phase, gas phase, smoke condensate or extract, e-liquid itself, or whole smoke
146 aerosol), the cell line used (*e.g.* lung epithelial cells, oral epithelial cells, oropharynx cells, or 'regulatory'
147 cells), or the mode of cell exposure [(*e.g.* submerged cell cultures or air-liquid interface (ALI) conditions)].
148 For these reasons, some of these cell treatment/exposure methods are not representative of an actual
149 human exposure to cigarette smoke or e-cig aerosols. In the Ames test, negative responses were
150 observed with e-liquid and e-cig aerosols (with nicotine and a range of flavorings) whereas positive results
151 were obtained concurrently with 3R4F smoke (Wieczorek et al., 2020). Similar negative results were

152 obtained with e-liquids and pad-collected aerosols of e-cigs, and positive results with pad-collected smoke
153 condensates of tobacco cigarettes (3R4F, 1R5F, Malboro gold) (Misra et al., 2014). Rudd *et al.* also
154 demonstrated that e-cig emission is not mutagenic under their tested conditions, unlike 3R4F cigarette
155 smoke (Rudd et al., 2020). Thorne *et al.* have carried out a study on *Salmonella typhimurium* strains TA98
156 and TA100 exposed at the air-agar interface to e-cig aerosols and showed no mutagenic activity in
157 contrast to 3R4F cigarette smoke (Thorne et al., 2016). The same authors also performed a mouse
158 lymphoma assay at the tk locus and *in vitro* micronucleus tests (on CHO, V79 and TK6 cells) with an e-
159 liquid, the e-cig aerosol matter captured from the same e-liquid, and the total particulate matter from a
160 3R4F cigarette. No mutagenic or genotoxic effect was observed for the e-liquid and its aerosol, in contrast
161 to 3R4F smoke (Thorne et al., 2019a, 2019b). All *in vitro* micronucleus tests reported in the literature were
162 negative (Misra et al., 2014; Rudd et al., 2020; Tellez et al., 2021; Thorne et al., 2019a; Wieczorek et al.,
163 2020), either with e-liquids, aerosols or condensates, except the one reported very recently by Tellez *et al.*
164 (2021) on e-cig aerosols (containing diverse flavoring product, with and without nicotine) in oral epithelial
165 cells. In contrast, in all these studies, traditional cigarette smoke (or total particulate matter or condensate)
166 induced chromosomal aberrations. Very recently, Tellez *et al.* demonstrated that 10 different e-cig
167 aerosols did not induce DNA damage, as measured by the *in vitro* comet assay, in oral epithelial cells,
168 unlike the 3R4F cigarette (Tellez et al., 2021). This result was not confirmed by several previously
169 published data. Khalil *et al.*, also using the *in vitro* comet assay, showed that e-cig aerosols cause DNA
170 damage in A549 lung cells exposed at the ALI (Khalil et al., 2021). Ganapathy *et al.* also reported that e-
171 cig aerosol extracts can induce significant increases in DNA damage (using the primer anchored DNA
172 damage detection assay), including 8-OHdG, on human oral and lung epithelial cells (Ganapathy et al.,
173 2017). Yu *et al.* observed increases in DNA strand breaks (as measured by the *in vitro* comet assay and
174 the γ H2AX immunostaining) after short- and long-term exposure (48 hours to 8 weeks) to e-cig aerosol
175 extracts, on several normal and cancerous cell lines (Yu et al., 2016). Finally, two studies performed on
176 A549 and/or BEAS-2B pulmonary cells exposed at the ALI to whole smoke from reference cigarettes
177 (M4A and/or 3R4F) reported the induction of DNA damage using the γ H2AX assay or the *in vitro* comet
178 assay (Garcia-Canton et al., 2014; Weber et al., 2013).

179 *In vivo* genotoxicity studies are much less numerous and less recent. Almost all of them have
180 been carried out on cigarette smoke and results show induction of micronuclei in bone marrow, peripheral
181 blood and lung of exposed rodents, as well as DNA damage in lung, stomach and liver cells (Balansky,

182 1999; Balansky et al., 2000; D'Agostini et al., 2001; Dalrymple et al., 2016, 2015; Nakamura et al., 2015;
183 Tsuda, 2000; Ueno et al., 2011). Some negative results have been also reported in the micronucleus and
184 the *Pig-a* mutation tests. In contrast, there is very little *in vivo* genotoxicity data on e-cigs with only two
185 published studies. Canistro *et al.* found that e-cig aerosol increased DNA damage and micronuclei
186 formation in peripheral blood of rats exposed for 4 weeks, and the collected urine of animals induced
187 reverse mutations in the Ames test (Canistro et al., 2017). Using the ³²P-postlabeling method, Lee *et al.*
188 showed that e-cig emission induced DNA adducts in lung, bladder and heart tissues of exposed mice (Lee
189 et al., 2018).

190

191 Because of a daily and prolonged consumption of e-cig by many users, it is now essential to
192 produce data on the mechanisms underlying the potential genotoxicity of e-cigs after long-term exposure.
193 In this context, the aim of our study was to investigate the *in vivo* genotoxic and mutagenic effects of e-cig
194 aerosols compared to traditional cigarette smoke. After nose-only exposure of BALB/c mice, the *in vivo*
195 genotoxic and mutagenic potential of smoke from conventional cigarette (3R4F) and emissions from a
196 "Modbox" e-cig model with 0.5 Ohms coil and set at 18W (Mb18W) or 30W (Mb30W) power were
197 assessed using (i) the *in vivo* comet assay in lung (primary target organ) and liver (systemic and most
198 active metabolizer organ), (ii) the *in vivo* micronucleus test in bone marrow and (iii) the *in vivo* *Pig-a* gene
199 mutation assay in peripheral blood (to identify possible systemic effects). The standard comet assay was
200 performed within the framework of subacute (4 days), subchronic (3 months) and chronic (6 months)
201 exposures. The micronucleus test and the *Pig-a* gene mutation assay, as markers of effects, were only
202 carried out for the 3- and 6-month exposures. For ethical and scientific reasons, these three tests were
203 applied to the same animals. In order to specifically determine oxidation-dependent DNA damage, we also
204 measured the pulmonary 8-OHdG content after subacute, subchronic and chronic exposures, and the
205 results were confirmed by performing a modified comet assay using the human 8-oxoguanine glycosylase
206 (hOGG1) after the 6-month treatment.

207

208 **2. MATERIALS AND METHODS**

209

210 **2.1. E-cigarettes, e-liquid and conventional cigarette**

211 Today there is a wide variety of e-cigs and e-liquids. As explained in our previous studies
212 (Beauval et al., 2019; Dusautoir et al., 2021), we chose the third generation “ModBox” model, used with
213 the “Air Tank” clearomiser equipped with a 0.5 Ω kanthal coil and with a partially closed air flow. For our
214 experiments, we chose two power settings for the Modbox model: a “low” power of 18W and a “high”
215 power of 30W. Both devices are from NHOSS[®] (Innova, Bondues, France). For the e-liquid, we chose the
216 best-selling NHOSS[®] brand containing 65% propylene glycol, 35% glycerine, 16 mg/mL nicotine and the
217 most common flavour, “blond tobacco”, representative of a standard e-liquid in accordance with the
218 French national organisation for standardisation (AFNOR) recommendations (AFNOR, Association
219 Française de Normalisation, 2015). Conventional 3R4F cigarettes were obtained from the University of
220 Kentucky (Lexington, KY, USA).

221

222 **2.2. Animal model**

223 Experiments were conducted on male BALB/c mice (Janvier Labs, Le Genest-Saint-Isle, France),
224 9 weeks old, 5 animals/group. This mouse strain is described as sufficiently sensitive to the chemical
225 induction of lung cancers (Meuwissen, 2005). Animal procedures were in agreement with European
226 directive 2010/63/EU for the protection of animals used for scientific purposes and obtained the Ethical
227 Committee on Animal Experimentation (CEEA 75) approval.

228

229 **2.3. Aerosol generation and mice exposure protocols**

230 To avoid chemical cross-contamination, two different pieces of equipment (exposure towers and
231 pipes) were used for e-cig and 3R4F exposures. Aerosols from e-cigs and 3R4F cigarette were generated
232 with an InExpose e-cigarette extension system on which we adapted the Modbox and a cigarette smoking
233 robot (SCIREQ[®], Emka technologies, Montreal, Quebec, Canada), respectively. Mice were exposed to
234 aerosols by a nose-only tower (InExpose system, SCIREQ[®], Emka technologies). In order to perform a
235 comparative study of the *in vivo* genotoxicity of the e-cig aerosols and tobacco cigarette smoke, all
236 products were tested with Health Canada Intense puff profile (55 mL puff volume, 2 s puff duration, 30 s
237 puff period).

238 Based on data from the literature and our preliminary study after a 4-day subacute exposure (data
239 not shown), three exposure protocols were applied in this study (Table 1). First, a subacute exposure for 4
240 days (4 treatments at 24-hour intervals for 30, 60 or 90 min/day for both e-cigs, and for 60 min/day for
241 3R4F) was performed as a preliminary toxicity assessment. Then, a 3-month subchronic and a 6-month
242 chronic exposure were realized (60 min/day, 5 days/week for e-cigs and 3R4F).

243 For each exposure schedule, one group was sham-exposed to fresh conditioned air (negative
244 control). Control groups with genotoxic reference compounds were also used for *in vivo* genotoxicity
245 studies (see part 2.5).

246 Animal body weights were recorded on Monday of each week while clinical signs were monitored
247 daily (data not shown).

248

249 **2.4. Chemical characterization of aerosols**

250 Chemical composition of aerosols from electronic and conventional cigarettes was assessed and
251 described in our previous study (Dusautoir et al., 2021). Chemical characterization analyses focused on
252 the quantification of nicotine and the identification and quantification of carbonyl compounds and PAHs
253 (see part 4).

254

255 **2.5. *In vivo* genotoxicity assessment**

256 The genotoxic/mutagenic potential of conventional and electronic cigarettes emissions was
257 assessed after subacute (4 consecutive days), subchronic (3 months) and chronic (6 months) exposures
258 by using a battery of three *in vivo* tests, namely the comet assay, the micronucleus assay and the *Pig-a*
259 gene mutation assay. These studies were carried out using an approach very similar to that of Good
260 Laboratory Practice (GLP). Tests, endpoints, target organs and treatment schedules are summarized in
261 Table 1.

262

263 **2.5.1. *In vivo* comet assay**

264 The *in vivo* comet assay was performed in isolated lung and liver cells under alkaline conditions
265 (pH>13) according to previously described protocol (Platel et al., 2020; Singh et al., 1988; Tice et al.,
266 2000; Witte et al., 2007) and in compliance with the OECD test guideline No. 489 (OECD, 2016a). At the
267 end of each exposure period, a positive control group was treated orally with methyl methanesulfonate

268 (MMS) [100 mg/kg body weight (b.w)/day for 2 consecutive days in sterile water]. For all groups (*i.e.*
269 treated and controls), tissues were collected once at 2-6 h after the last treatment. For the 6-month
270 exposure time, slight modifications were added (use of hOGG1) to specifically detect oxidative DNA
271 damage, based on Collins' and Smith's procedures (Collins et al., 1993; Smith et al., 2006). 750 randomly
272 selected cells per group (*i.e.* 50 cells per slide, 3 slides per animal, 5 animals per group) were analysed for
273 DNA fragmentation scoring using the Comet Assay IV Image Analysis System, version 4.11 (Perceptive
274 Instruments Ltd, Suffolk, United Kingdom). DNA damage was expressed as percentage of DNA in the tail
275 (% tail intensity) (Burlinson *et al.*, 2007; Lovell and Omori, 2008).

276

277 **2.5.2. *In vivo* micronucleus test**

278 The *in vivo* micronucleus test was performed in the bone marrow of treated mice in compliance
279 with the OECD test guideline No. 474 (OECD, 2016b). A positive control group was treated orally with
280 MMS [100 mg/kg b.w/day (x2 days) in sterile water] (see part 2.5.1). The protocol has been previously
281 described (Platel et al., 2020). Two slides per animal were prepared. For the determination of genotoxicity,
282 slides were blindly scored by microscopy for the number of polychromatic erythrocytes (PCE) (2000 PCE
283 per slide, *i.e.* 4000 PCE per animal) having one or more Howell-Jolly bodies (micronucleated
284 polychromatic erythrocytes, MNPCE). For the determination of cytotoxicity, the
285 polychromatic/normochromatic erythrocyte ratio (PCE/NCE) was determined from the microscopic
286 examination of at least 500 erythrocytes per slide (*i.e.* 1000 erythrocytes per animal).

287

288 **2.5.3. *In vivo* *Pig-a* gene mutation assay**

289 The quantification of *in vivo* *Pig-a* (phosphatidylinositol glycan, class A) gene mutation (Bryce et
290 al., 2008; Dobrovolsky et al., 2010; Kimoto et al., 2011) was performed with the MutaFlow^{PLUS} Kit Mouse
291 Blood (Litron, Rochester, New York) as previously described (Platel et al., 2020). According to the kinetics
292 for mutant phenotype cells appearance in circulation and ease of scoring, one month before the end of
293 each exposure period, a positive control group was treated orally with ethyl-nitrosourea (ENU) [40 mg/ kg
294 b.w/day for 3 consecutive days in sterile water]. Blood samples were collected after 3 and 6 months of
295 exposure. The incidence of *Pig-a* mutation per animal was expressed as the number of CD24-negative
296 red blood cells (RBC) per one million RBC, and as the number of CD24-negative reticulocytes (RET) per

297 one million RET, using a FACSCanto II flow cytometer (BD Biosciences) running FACSDiva™ v7.0
298 software. The percentage of RET was also established for cytotoxicity assessment.

299

300 **2.6. 8-hydroxy-2'-deoxyguanosine (8-OHdG) assay**

301 8-OHdG level was measured in the genomic DNA of mouse lung tissues following 4 days, 3 months or 6
302 months of exposure to either electronic or conventional cigarette aerosols. Genomic DNA was extracted
303 using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France) following manufacturer's
304 recommendations. Extracted DNA was pre-treated with P1 nuclease using the reagents from Wako 8-
305 OHdG Assay Preparation (Wako, Tokyo, Japan). This step permits to digest the DNA down to the single
306 nucleotide level. 8-OHdG level were then determined using a competitive enzyme-linked immunosorbent
307 assay (ELISA): Oxiselect™ Oxidative DNA Damage Kit (Cell Biolabs, San Diego, CA), according to the
308 manufacturer's recommendations. Results were expressed as fold-change (\pm SD) relative to the 8-OHdG
309 level in control mice arbitrarily set at a value of 1.

310

311 **2.7. Statistical Analysis**

312 All statistical analyses were performed with GraphPad InStat® Software (version 3.10). For each
313 test, differences between groups (*i.e.* between each concentration vs. the respective negative control) with
314 $p < 0.05$ were considered statistically significant.

315 The Mann-Whitney U-test was used for the comet assay, the micronucleus test (for the frequency of
316 MNPCE) and the 8-OHdG content. The Student's t test was used for the statistical comparison for the
317 PCE/NCE ratio (micronucleus test). The Dunnett's t-test (pair-wise comparison) was performed for the
318 *Pig-a* gene mutation assay.

319

320 3. RESULTS

321 Results of the *in vivo* tests are summarized in Table 2. For each test, concurrent negative controls
322 (animals sham-exposed to fresh conditioned air) were within the range of current observed values and
323 concurrent positive controls induced responses that are comparable to the historical positive control data
324 (data not shown) and produced a statistically significant increase compared with the negative control. The
325 validity criteria for the tests were considered as fulfilled.

326

327 3.1. Subacute exposure (4-day treatment)

328 The genotoxic potential of electronic and conventional cigarettes was investigated in the *in vivo*
329 comet assay on isolated lung and liver cells of mice after a subacute exposure (4 treatments at 24-hour
330 intervals for 90 min/day for both e-cigs, and for 60 min for 3R4F). Results of the means of medians of
331 percentage of tail intensity (TI) are given in Figure 1. Under tested conditions, no increase in DNA strand
332 breaks was observed in the two selected organs, for both conventional and electronic cigarettes.

333 Regarding the levels of 8-OHdG in mouse lung tissues, exposure to cigarette smoke for 60 min, 4
334 days in a row, induced a significant increase relative to air-exposed mice (1.6 fold-change) (Figure 4A).
335 For e-cigs, subacute exposure to Mb18W aerosol induced no change in 8-OHdG levels regardless of the
336 duration of exposure (*i.e.* 30, 60 or 90 min), whereas exposures to Mb30W emissions for 60 min and 90
337 min induced a statistically significant increase compared to the control (1.5 and 1.6 fold-changes) (Figure
338 4A).

339

340 3.2. Subchronic exposure (3-month treatment)

341 Results of genotoxicity/mutagenicity assessment after the 3 months subchronic exposure of mice
342 (60 min/day, 5 days/week) are presented in Figure 2.

343 For both e-cigs and the conventional cigarette, no statistically significant increase in the level of
344 DNA damage was observed, in either the liver (Figure 2A) or the lung (Figure 2B). The highest TI was
345 obtained with Mb30W in the liver (2.3 % vs. 1.69 % for the negative control).

346 Regarding the frequency of MNPCE, no significant increase was found in animals exposed to
347 Mb18W (0.75 ‰), Mb30W (1.13 ‰) or 3R4F (0.60 ‰) emissions when compared to the control group
348 (0.55 ‰) (Figure 2C). The ratio PCE/NCE was not significantly affected by exposure to e-cig aerosols and

349 3R4F cigarette smoke, indicating the absence of cytotoxic effects (a very slight decrease but non-
350 statistically significant was observed with 3R4F).

351 The frequencies of mutants RET (highest value: 2.92×10^{-6} for 3R4F) and mutants RBC (highest
352 value: 3.42×10^{-6} for 3R4F) did not show statistically significant increase in the animals exposed to
353 Mb18W, Mb30W or 3R4F emissions when compared to the control group exposed to air (RET = 1.44×10^{-6}
354 and RBC = 2.08×10^{-6}) (Figure 2D). The percentage of RET is the ratio of newly formed RNA-positive
355 erythrocyte relative to all erythrocytes, and is used as a measure of bone marrow cytotoxicity. E-cig and
356 conventional cigarette exposed mice did not exhibit significant changes in % RET after a 3-month
357 exposure, thus confirming the absence of toxicity.

358 After 3 months of exposure to Mb30W aerosol and 3R4F smoke, 8-OHdG quantity assessment in
359 DNA of mouse lung tissues showed a statistically significant increase compared to control (1.8 and 2.0
360 fold-changes, respectively) while an exposure to Mb18W emissions induced no change (Figure 4B).

361

362 **3.3. Chronic exposure (6-month treatment)**

363 Results obtained after the 6-month chronic exposure (60 min/day, 5 days/week) are presented in
364 Figure 3.

365 In the standard comet assay, no increase in DNA strand breaks was observed for both
366 conventional and electronic cigarettes. On the contrary, with the hOGG1-modified comet assay,
367 statistically significant increases ($p < 0.05$) in TI were observed for Mb30W and 3R4F in the liver (15.15 %
368 and 11.46 %, respectively, vs. 1.52 % for the negative control) (Figure 3A) and in the lung (34.96 % and
369 30.59 %, respectively, vs. 11.57 % for the negative control) (Figure 3B), indicating oxidative DNA damage
370 induction.

371 Under tested conditions, no induction of MN formation was observed in mice exposed to e-cigs
372 18W, 30W or 3R4F cigarette aerosols ($< 0.8 \%$). No decrease of the ratio PCE/NCE was observed (Figure
373 3C).

374 No statistically significant increase in mutant frequencies of RBC (highest value: 0.37×10^{-6} cells
375 for 3R4F) and RET (highest value: 0.47×10^{-6} cells for 3R4F) was observed whatever the types of
376 cigarette compared to the control group (RET mutant frequency = 1.33×10^{-6} and RBC mutant frequency
377 = 0.65×10^{-6}) (Figure 3D). The % RET was not significantly affected indicating the absence of toxic effects
378 in the bone marrow at this exposure level.

379 Consistent with subacute and subchronic exposures, a 6-month exposure to Mb18W aerosol
380 induced no change in the level of 8-OHdG in the lung tissue DNA of mice compared to air-exposed mice,
381 whereas Mb30W aerosol and 3R4F smoke induced a statistically significant increase (1.2- and 1.4-fold,
382 respectively) (Figure 4C).

383

384 4. DISCUSSION

385 Electronic nicotine delivery systems are considered by public opinion to be less harmful than
386 traditional cigarettes, and are currently used as a smoking cessation aid. Paradoxically, there is a lack of
387 long-term *in vivo* studies on their health effect, thus their safety cannot be claimed. To fill this gap, we
388 carried out a comprehensive assessment of the *in vivo* genotoxicity and mutagenicity of an e-cig model
389 set to two different power levels (18W and 30W) and of conventional cigarette. The conditions of animal
390 exposure, in terms of route (pulmonary), mode (nose-only), time (short and long-term treatment) and puff
391 profile (Health Canada Intense profile), were designed to be as close as possible to human vaping
392 conditions.

393

394 Under our experimental conditions, whatever the duration of animal exposure, 3R4F cigarette and
395 e-cigs at both powers did not induce an increase in DNA strand breaks in lung and liver cells, as
396 measured by the standard comet assay. This result may seem in contradiction with the study carried out
397 by Canistro *et al.* in which e-cig aerosol produced DNA damage in leukocytes of whole-body exposed rats
398 (Canistro *et al.*, 2017). However, as the authors themselves stated, their data should be analysed with
399 caution as the exposure conditions used [animals were submitted to 11 cycles (puff: 6s on, 5s off, 6s
400 on)/day, 5 days/week, for 4 weeks] did not reflect actual human exposure to e-cig aerosols. Their aim was
401 to characterize a hazard and perhaps the use of too high doses may explain the induction of non-specific
402 DNA damage. Other published data have also shown positive results in the *in vivo* comet assay on
403 stomach, liver and/or lung with cigarette smoke (Tsuda, 2000; Ueno *et al.*, 2011).

404 On the other hand, our results showed that only Mb30W and 3R4F aerosols induced a statistically
405 significant increase in 8-OHdG formation in the lung of exposed mice after 4 days, 3 months and 6 months
406 of exposure. At the end of our study (*i.e.* for the 6-month exposure) we decided to confirm this result by
407 using a modified protocol for the comet assay. Indeed, we used the repair endonuclease hOGG1 to better
408 characterize the mechanism of genotoxicity of e-cig emissions and conventional cigarette smoke. The
409 hOGG1-modified comet assay is a useful tool to increase both the sensitivity and the specificity of the test
410 and thus provide first elements of the oxidizing mode of action of test compounds (Platel *et al.*, 2011). The
411 corresponding results were consistent with the 8-OHdG measurement since only Mb30W and 3R4F
412 aerosols induced significant oxidative DNA damage in the lung and the liver of exposed mice. Our findings
413 are also in line with our previous study (Dusautoir *et al.*, 2021) and with reviews reporting that exposure to

414 e-cig aerosols is related to oxidative stress (Cao et al., 2021; Merez-Sadowska et al., 2020; Polosa et al.,
415 2019; Wang et al., 2019). Interestingly, Dalrymple *et al.* also showed, after 5 days of nose-only exposure
416 of rats to 3R4F cigarette smoke, an increase in oxidative DNA damage in alveolar type II lung cells
417 exclusively by using the FPG-modified comet assay (*i.e.* no DNA damage was observed with the classical
418 protocol without FPG) (Dalrymple et al., 2015). The authors also found oxidative DNA damage after 3 and
419 6 weeks of exposure (Dalrymple et al., 2016).

420 Very recently, we have carried out a comparison of the chemical composition of aerosols from
421 Mb18W, Mb30W and 3R4F (Dusautoir *et al.*, 2021). We showed that increasing the power of the e-cig can
422 induce an increase in the amount of toxic compounds in the aerosol (by puff, Mb18W emitted 6.9% and
423 51.4% less total PAHs and carbonyl compounds, respectively, than Mb30W). It has been previously
424 demonstrated that higher power leads to higher carbonyls compounds production due to higher coil
425 temperature (up to 300°C) and thus the thermal degradation of e-liquid and that, secondarily, the
426 increased level of carbonyl compounds results in the formation of ROS (Dusautoir et al., 2021; Geiss et
427 al., 2016; Haddad et al., 2019; Kosmider et al., 2014; Zhao et al., 2018). Our results are thus consistent
428 with these explanations since in our study oxidative DNA damage was observed only with Mb30W.

429 Noteworthy, we observed an almost similar response between the 3R4F cigarette and the Mb30W
430 e-cig. It is difficult, if not impossible, to define precisely which toxic substance(s) is (are) responsible for
431 the genotoxic effect observed in each case. The use of predictive toxicity methods (*i.e.* *in silico* models)
432 would be an interesting tool for this purpose as an alternative approach to experimental testing. In the
433 study performed by Barhdadi *et al.*, a genotoxic alert was identified by (Q)SAR models for 60 flavoring
434 substances identified among the 129 e-liquids tested (Barhdadi et al., 2021). Based on information
435 collected from EU databases 5 flavoring substances of genotoxic concern were identified (estragole,
436 safrole, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone, furylmethylketon and trans-hexenal) and 4 substances
437 (2,3-butanedione, 2,3-pentanedione, isolekene and β -phellandrene) gave positive result in at least one *in*
438 *vitro* test (Ames and/or *in vitro* micronucleus test). Similarly, Kang *et al.* used (Q)SAR models to predict
439 DNA adducts formation by flavor chemicals found in e-liquid and e-cig aerosols (Kang and Valerio, 2020).
440 Two chemical classes were identified, alkenylbenzenes (including estragole and eugenol) and aldehydes
441 (including acrolein, glyoxal and methylglyoxal), well known to be produced in cigarette smoke and e-cig
442 aerosol (Beauval et al., 2019; Bekki et al., 2014; Dusautoir et al., 2021; Hutzler et al., 2014; Khlystov and
443 Samburova, 2016; Peace et al., 2018).

444 Lee *et al.*, as a step towards understanding the carcinogenicity of e-cig aerosols, demonstrated
445 that nicotine (noncarcinogenic in animals) can be nitrosated, metabolized, and further transformed into
446 methyldiazohydroxide (MDOH) and aldehydes in lung, bladder, and heart tissues of mice (Lee *et al.*,
447 2018). They found that aldehydes and MDOH induced DNA adducts and also decreased DNA repair.
448 Interestingly, we previously showed that the level of nicotine delivered in the aerosols is much lower for
449 Mb18W (60 µg/puff) than for Mb30W (137 µg/puff) and 3R4F (95 µg/puff) (Dusautoir *et al.*, 2021).
450 Therefore, it can be assumed that the level of DNA adducts to be formed could be less for Mb18W which
451 is consistent with our results.

452
453 In addition, our results revealed that both traditional cigarette smoke and e-cig aerosol induced no
454 biologically or statistically significant increases in chromosomal aberrations and gene mutations, whatever
455 the duration of exposure. Therefore, they are considered having no mutagenic activity under our
456 experimental conditions. These results, although at first sight surprising, are fully in line with those of
457 Dalrymple *et al.* (2016). In their study, rats were nose-only exposed to 3R4F cigarette (1h or 2h/day, 5
458 days/week) for 3 and 6 weeks. Blood was collected only at the 6-week timepoint and results showed that
459 *Pig-a* gene mutations and micronucleus frequencies were not significantly increased (as mentioned
460 above, positive results were obtained in the modified comet assay). Others have also obtained negative
461 results in the *in vivo* micronucleus test on bone marrow or peripheral blood following nose-only cigarette
462 exposure (Schramke *et al.*, 2014; Van Miert *et al.*, 2008). For e-cig, no data was found in the literature
463 regarding the assessment of its *in vivo* mutagenicity, with the exception of the study by Canistro *et al.*
464 (2017) that showed micronuclei formation in reticulocytes of rats whole-body exposed to e-cig aerosol.
465 However, as explained above, their data should be compared with ours with caution because the
466 exposure conditions they used were not intended to reflect actual human exposure to e-cig emission but
467 rather to characterize a hazard. Indeed, in our study, the absence of mutagenic effect of reference
468 cigarette smoke and e-cig aerosols, while it is well known that they are composed of carcinogenic
469 substances, suggest that the experimental conditions we implemented, although realistic, may not be high
470 enough to reach a level of exposure in bone marrow and blood to induce a positive response in the
471 micronucleus and *Pig-a* tests, respectively. Furthermore, as already mentioned by Dalrymple *et al.* (2016)
472 it is possible that cigarette and e-cig do not induce mutagenic effect in organs other than the respiratory
473 system (*i.e.* the first tissue of contact and target organ of tobacco products). Another important point that

474 may explain the negative results is the sensitivity of the tests. Although the *in vivo* micronucleus test in
475 bone marrow or peripheral blood is traditionally the most used *in vivo* test in the first instance, it is known
476 to have a poor sensitivity of about 40-50% (Benigni et al., 2010; Kirkland and Speit, 2008; Morita et al.,
477 2016). As there is no single 'ideal' test for detecting clastogenic, aneugenic and mutagenic genetic events,
478 it is common to use a combination of several tests (different genotoxic endpoints), as we did in our study,
479 to increase sensitivity without reducing specificity. The *Pig-a* test is known for its remarkable sensitivity to
480 mutagenic agents (Gollapudi et al., 2015) and its relatively sensitivity to clastogens (Bhalli et al., 2013).
481 Ideally, these tests should have been carried out on the lung and liver (*i.e.* on the target organs), but for
482 methodological reasons this is not feasible. Despite an inter-laboratory study showed that the combination
483 of the comet, micronucleus and *Pig-a* assays, using the same animals, may be a robust strategy for
484 evaluating *in vivo* genotoxicity (Chung et al., 2018), it would have been relevant to perform an *in vivo* gene
485 mutation test on the target organs (*i.e.* liver and lung) using transgenic animals. Indeed, transgenic rodent
486 gene mutation tests have the ability to detect and quantify mutations in virtually all somatic tissues
487 (Gingerich et al., 2014; Lambert et al., 2005; OECD, 2020). However, these tests are complex, currently
488 expensive and not widely available.

489

490 5. CONCLUSION

491 The e-cig was initially developed as an alternative to conventional cigarette although there is
492 insufficient data to assess its long-term safety for human health. In this context, our study was
493 implemented with the aim of comparing the *in vivo* genotoxic and mutagenic potential of two low- and
494 high-power e-cigs and the traditional cigarette, after subacute (4 days), subchronic (3 months) and chronic
495 (6 months) exposure. In order to be as close as possible to human exposure conditions, animals were
496 exposed to realistic doses of e-cig and cigarette emissions (*i.e.* Health Canada Intense puff profile) *via* the
497 pulmonary route (nose-only). Under these experimental conditions, the main result of our study is that
498 both 3R4F and Mb30W induce oxidative DNA damage in lung and liver, demonstrating that high-power e-
499 cig should be considered as “hazardous material” as traditional cigarette, whereas e-cig at low power
500 setting seems to be devoid of *in vivo* genotoxic effect. These differences in results between Mb18W and
501 Mb30W are probably attributable to lower concentrations of toxic substances (mainly carbonyls
502 compounds) in low power e-cig aerosols, as previously described. Moreover, micronuclei and *Pig-a* gene
503 mutation were not detected in reticulocytes. This suggests that our experimental conditions, although
504 realistic, may not be sufficient to reach a level of exposure in bone marrow and blood to induce a positive
505 response. This also raises the question of the sensitivity of these two tests in organs other than the target
506 organ (here the lung). It is important to underline the originality of our work which is based on a complete
507 study of the *in vivo* genotoxic/mutagenic potential of e-cig. Finally, our work could be completed by
508 assessing gene mutations in the target organs (*i.e.* liver and lung) using the transgenic rodent mutation
509 assay. It would also be interesting to study other non-genotoxic endpoints involved in the potential
510 carcinogenesis of e-cig such as epigenetic alterations. All these data could lead to a better regulation of
511 these new alternatives to conventional cigarettes.

512

513 **6. FUNDING**

514 This work benefited grant from the French Institute of Cancer (INCa): Contracts n°INCa_11505.

515

516 **7. DECLARATION OF COMPETING INTEREST**

517 The authors declare that they have no conflict of interest with tobacco or e-cig industries.

518

519 **8. ACKNOWLEDGMENTS**

520 We thank Dr H el ene Bauderlique from the Bioluminescence Imaging Center Lille for facility access to the
521 cytometer BD FACSCanto II (BICeL-campus Pasteur, 1 rue du Prof Calmette, 59019 Lille, France).

522

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544

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873 **TITLE AND LEGEND OF TABLES AND FIGURES**

874

875 **Figure 1. Results of the *in vivo* comet assay after subacute exposure of mice to e-cig and**
876 **conventional cigarette aerosols.**

877 Animals (n=5) were exposed to conventional cigarette (3R4F) smoke for 60 min/day or to e-cig (Mb18W
878 and Mb30W) emissions for 90 min/day, for 4 consecutive days. The negative control group was exposed
879 to air. MMS [(100 mg/kg b.w./day)x2] was used as positive control. The level of DNA fragmentation on
880 liver **(A)** and lung **(B)** cells is expressed as the mean of medians of % of tail DNA intensity (\pm SD). **
881 $p < 0.01$ (Mann-Whitney U-test).

882

883

884 **Figure 2. Results of *in vivo* genotoxicity/mutagenicity assessment after subchronic exposure of**
885 **mice to e-cig and conventional cigarette aerosols.**

886 Animals (n=5) were exposed to conventional cigarette (3R4F) smoke or to e-cig (Mb18W and Mb30W)
887 emissions for 60 min/day, 5 days/week, for 3 months. The negative control group was exposed to air.
888 MMS [(100 mg/kg b.w./day)x2] and ENU [(40 mg/kg b.w./day)x3] were used as positive controls. **(A-B)**
889 The level of DNA fragmentation is expressed as the mean of medians of % of tail DNA intensity (\pm SD). **(C)**
890 The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic
891 erythrocytes (MNPCE) per 1000 cells (\pm SD). The polychromatic erythrocytes (PCE) / normochromatic
892 erythrocytes (NCE) ratio is used as a measure of bone marrow cytotoxicity. **(D)** The gene mutations
893 frequency is expressed as the number of red blood cells (RBC) or reticulocytes (RET) per 10^6 cells (\pm SD).
894 Toxicity in bone marrow was measured by % RET. * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney U-test for the
895 comet assay and the micronucleus test, Dunnett's t-test for the *Pig-a* test).

896

897

898 **Figure 3. Results of *in vivo* genotoxicity/mutagenicity assessment after chronic exposure of mice**
899 **to e-cig and conventional cigarette aerosols.**

900 Animals (n=5) were exposed to conventional cigarette (3R4F) smoke or to e-cig (Mb18W and Mb30W)
901 emissions for 60 min/day, 5 days/week, for 6 months. The negative control group was exposed to air.
902 MMS [(100 mg/kg b.w./day)x2] and ENU [(40 mg/kg b.w./day)x3] were used as positive controls. **(A-B)**

903 The level of DNA fragmentation is expressed as the mean of medians of % of tail DNA intensity (\pm SD). **(C)**
904 The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic
905 erythrocytes (MNPCE) per 1000 cells (\pm SD). The polychromatic erythrocytes (PCE) / normochromatic
906 erythrocytes (NCE) ratio is used as a measure of bone marrow cytotoxicity. **(D)** The gene mutations
907 frequency is expressed as the number of red blood cells (RBC) or reticulocytes (RET) per 10^6 cells (\pm SD).
908 Toxicity in bone marrow was measured by % RET. * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney U-test for the
909 comet assay and the micronucleus test, Dunnett's t-test for the *Pig-a* test).

910

911

912 **Figure 4. Results of *in vivo* lung 8-OHdG assessment in mice after acute, subchronic and chronic**
913 **exposure to e-cig and conventional cigarette aerosols.**

914 Animals were exposed to conventional cigarette (3R4F) or to e-cig (Mb18W and Mb30W) emissions for
915 30, 60 or 90 min/day for 4 days for subacute exposures ($n = 5$) and for 60 min/day, 5 days/week for 3 or 6
916 months for subchronic and chronic exposures ($n = 8$), respectively. The control group was exposed to air.
917 The level of 8-OHdG is expressed as fold-change relative to the level found in control mice (\pm SD)
918 measured using a competitive ELISA assay following 4 days **(A)**, 3 months **(B)** or 6 months **(C)** of
919 exposure. * $p < 0.05$ (Mann-Whitney U-test).

920

921

922 **Table 1. Summary of *in vivo* genotoxic/mutagenic tests performed.**

923 For subacute exposure, mice received 4 treatments at 24-hour intervals for 90 min (and for 30 and 60 min
924 for the 8-OHdG assay) for e-cigs, and for 60 min for conventional cigarette. For subchronic (3 months) and
925 chronic (6 months) exposures, animals were exposed to e-cig or 3R4F cigarette emissions for 60 min, 5
926 times a week. X: test performed.

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929 **Table 2. Summary of *in vivo* genotoxicity/mutagenicity tests results.**

930 For subacute exposure, mice were exposed 4 times at 24-hour intervals for 90 min (and for 30 and 60 min
931 for the 8-OHdG assay) to Mb18W and Mb30W aerosols, and for 60 min to 3R4F smoke. For subchronic (3

932 months) and chronic (6 months) exposures, animals were exposed 60 min/day, 5 days/week, to Mb18W,
933 Mb30W and 3R4F aerosols. n.a.: not assessed; -: negative result; +: positive result.

FIGURE 1

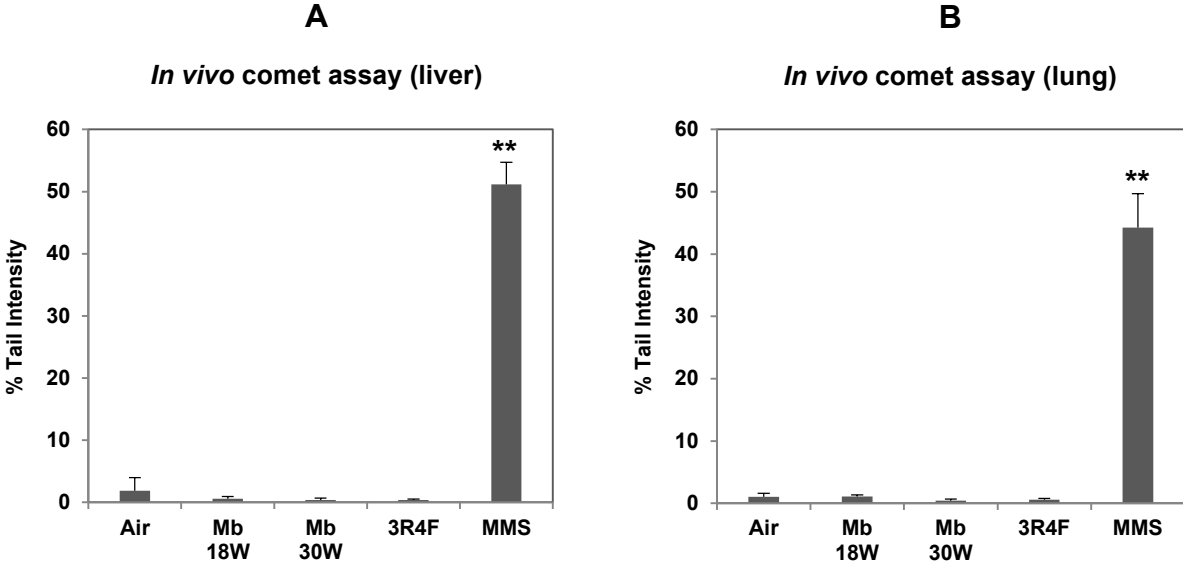


FIGURE 2

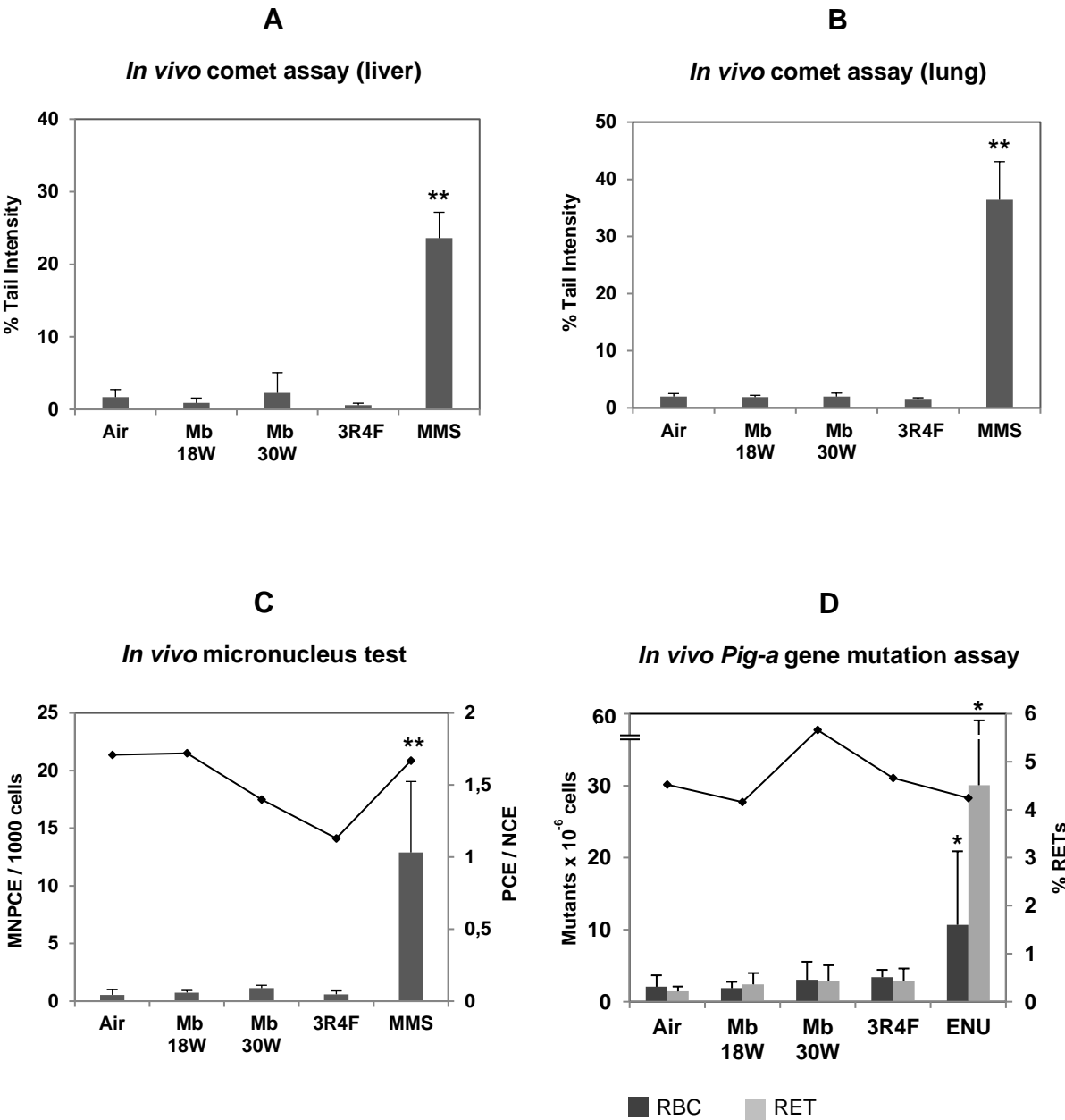
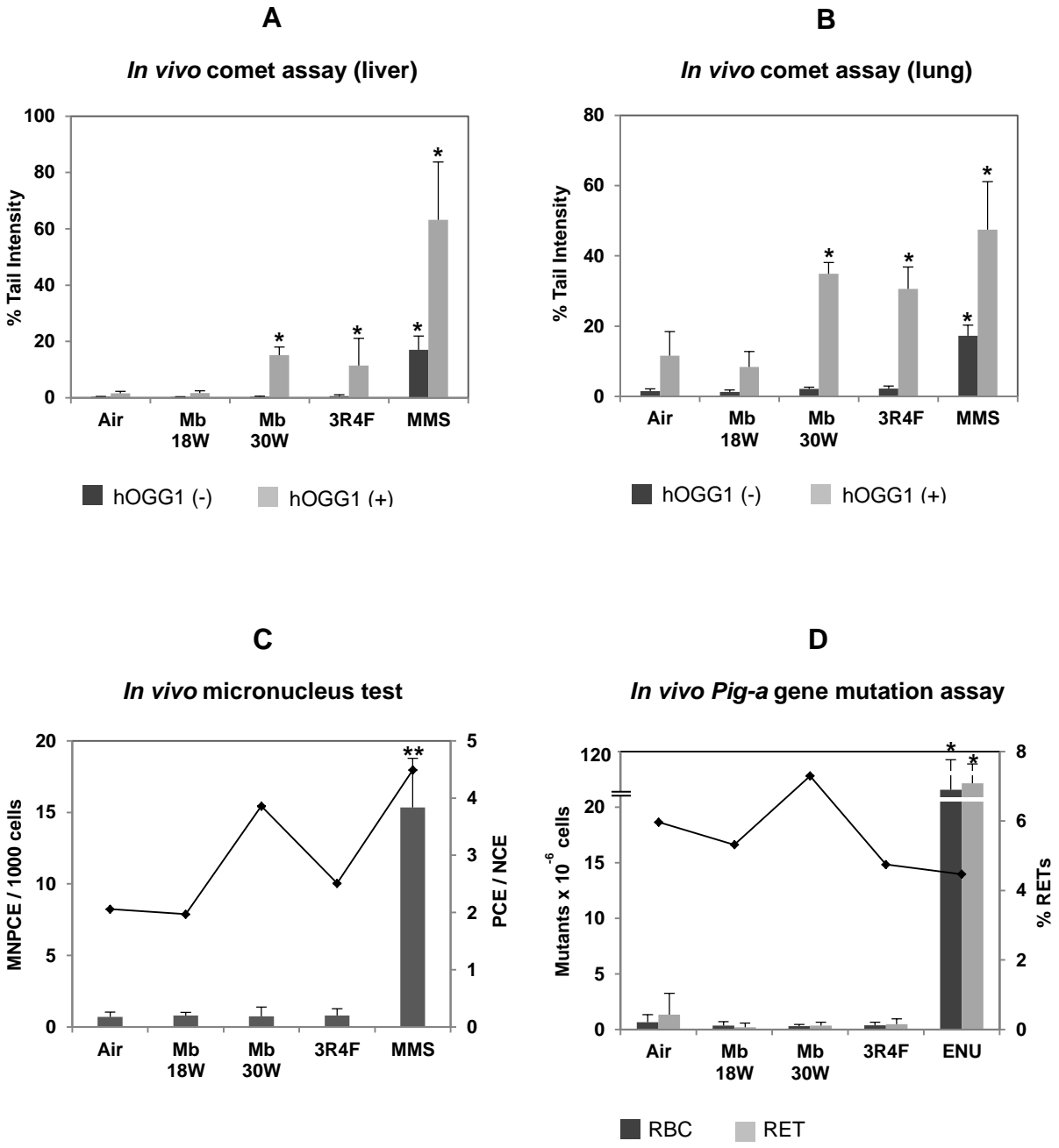
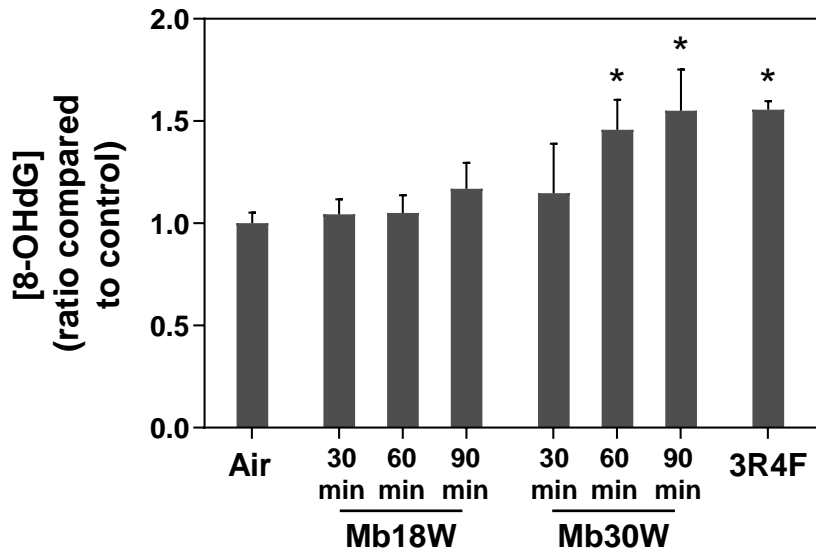


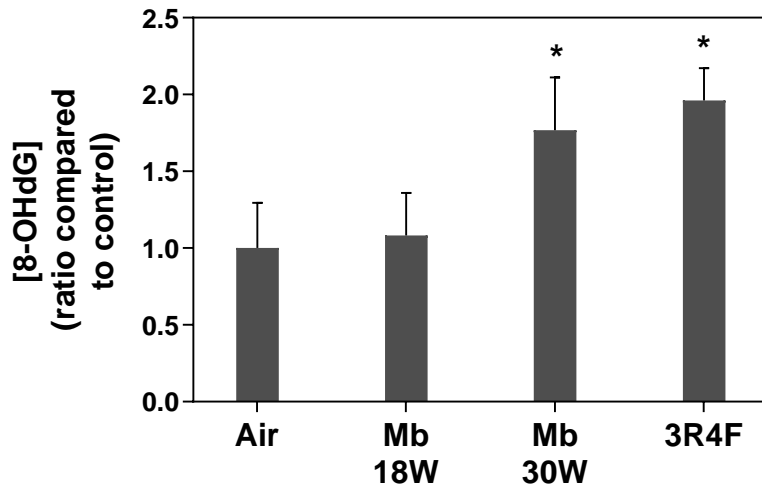
FIGURE 3



A Subacute



B Subchronic



C Chronic

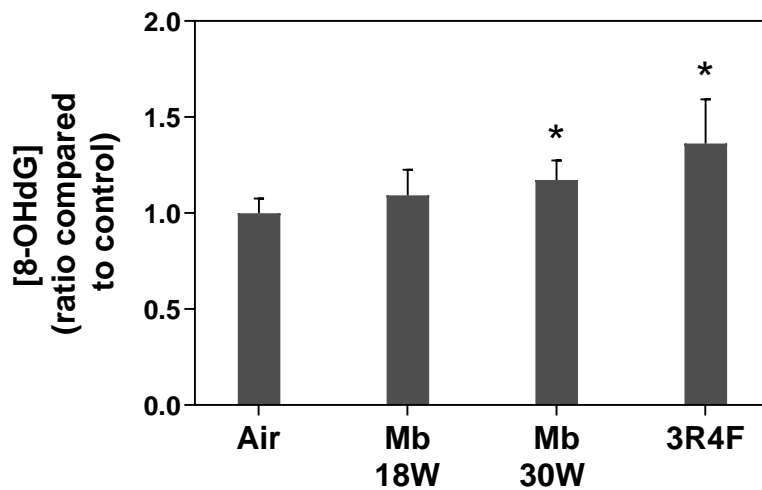


TABLE 1

<i>In vivo</i> tests	End-points	Target organs	Exposure time		
			4 days	3 months	6 months
Standard comet assay	Primary DNA damage	Liver, lung	X	X	X
Micronucleus test	Chromosomal aberrations	Bone marrow		X	X
<i>Pig-a</i> gene mutation assay	Gene mutations	Erythrocytes		X	X
hOGG1-modified comet assay	Oxidative DNA damage	Liver, lung			X
8-OHdG assay	8-OHdG	Lung	X	X	X

TABLE 2

	4 days			3 months			6 months		
	Mb 18W	Mb 30W	3R4F	Mb 18W	Mb 30W	3R4F	Mb 18W	Mb 30W	3R4F
Standard comet assay	-	-	-	-	-	-	-	+	+
Micronucleus test	n.a.	n.a.	n.a.	-	-	-	-	-	-
<i>Pig-a</i> assay	n.a.	n.a.	n.a.	-	-	-	-	-	-
hOGG1-modified comet assay	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	+	+
8-OHdG assay	-	+	+	-	+	+	-	+	+

Graphical abstract

