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## Study of in vitro and in vivo genotoxic effects of air pollution fine (PM<sub>2.5-0.18</sub>) and quasi-ultrafine (PM<sub>0.18</sub>) particles on lung models

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1 **Study of *in vitro* and *in vivo* genotoxic effects of air pollution fine (PM<sub>2.5-0.18</sub>) and**  
2 **quasi-ultrafine (PM<sub>0.18</sub>) particles on lung models**

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40 **ABSTRACT**

41 Air pollution and particulate matter (PM) are classified as carcinogenic to humans . Pollutants evidence for  
42 public health concern include coarse (PM<sub>10</sub>) and fine (PM<sub>2.5</sub>) particles. However, ultrafine particles (PM<sub>0.1</sub>)  
43 are assumed to be more toxic than larger particles, but data are still needed to better understand their  
44 mechanism of action.

45 In this context, the aim of our work was to investigate the *in vitro* and *in vivo* genotoxic potential of fine  
46 (PM<sub>2.5-0.18</sub>) and quasi ultra-fine (PM<sub>0.18</sub>) particles from an urban-industrial area (Dunkirk, France) by using  
47 comet, micronucleus and/or gene mutation assays. *In vitro* assessment was performed with 2 lung  
48 immortalized cell lines (BEAS-2B and NCI-H292) and primary normal human bronchial epithelial cells  
49 (NHBE) grown at the air-liquid interface or in submerged conditions (5 µg PM/cm<sup>2</sup>). For *in vivo*  
50 assessment, tests were performed after acute (24 hours, 100 µg PM/animal), subacute (1 month, 10 µg  
51 PM/animal) and subchronic (3 months, 10 µg PM/animal) intranasal exposure of BALB/c mice.

52 *In vitro*, our results show that PM<sub>2.5-0.18</sub> and PM<sub>0.18</sub> induced primary DNA damage but no chromosomal  
53 aberrations in immortalized cells. Negative results were noted in primary cells for both endpoints. *In vivo*  
54 assays revealed that PM<sub>2.5-0.18</sub> and PM<sub>0.18</sub> induced no significant increases in DNA primary damage,  
55 chromosomal aberrations or gene mutations, whatever the duration of exposure.

56 This investigation provides initial answers regarding the *in vitro* and *in vivo* genotoxic mode of action of  
57 PM<sub>2.5-0.18</sub> and PM<sub>0.18</sub> at moderate doses and highlights the need to develop standardized specific  
58 methodologies for assessing the genotoxicity of PM. Moreover, other mechanisms possibly implicated in  
59 pulmonary carcinogenesis, e.g. epigenetics, should be investigated.

60

61

62 **KEYWORDS**

63 Fine and ultrafine particles

64 Air pollution

65 Human lung cell models

66 Animal model

67 Genotoxicity

68

## 69 1. INTRODUCTION

70 Air pollution levels remain dangerously high in many parts of the world. Both short- and long-term  
71 exposure to air pollutants have been associated with health impacts in children and adults (Burns *et al.*,  
72 2014; Lim *et al.*, 2012; Loomis *et al.*, 2013; R ckerl *et al.*, 2011; Schraufnagel *et al.*, 2019; World Health  
73 Organization, 2016). According to the World Health Organization, in 2016, around 8 million people die  
74 every year from exposure to polluted air (World Health Organization, consulted on September 2019). Of  
75 those deaths, around 4.2 million are attributable solely to ambient (outdoor) air pollution, mainly from heart  
76 disease, stroke, chronic obstructive pulmonary disease (COPD), lung cancer and acute respiratory  
77 infections in children. In 2016, 91% of the world population was living in places where the WHO air quality  
78 guidelines levels were not met (World Health Organization, consulted on September 2019). A new study  
79 published recently shows that the scale of the phenomenon could be worse. Indeed, ambient air pollution  
80 could be twice as deadly as what the scientific community admitted until then; it would thus cause 8.8  
81 million deaths a year in the world (Lelieveld *et al.*, 2019). Pollutants with the strongest evidence for public  
82 health concern include coarse (PM<sub>10</sub>, *i.e.* particulates of median aerodynamic diameter  $\leq 10 \mu\text{m}$ ) and fine  
83 particulate matter (PM<sub>2.5</sub> or FPs, *i.e.* particulates of median aerodynamic diameter  $\leq 2.5 \mu\text{m}$ ). PM is of  
84 major toxicological interest due to its great heterogeneity. PM consists in a complex mixture made of an  
85 inorganic core (ions, metals, inorganic carbonaceous species) coated with organic compounds such as  
86 polycyclic aromatic hydrocarbons (PAHs), semi-volatile organic compounds, polychlorobiphenyls, furans,  
87 dioxins, and micro-organisms (bacteria and fungi) (Harrison and Yin, 2000). Ultrafine PM is capable of  
88 penetrating and lodging deep inside the lungs and entering the bloodstream causing cardiovascular,  
89 cerebrovascular and respiratory effects (Cho *et al.*, 2018). The adverse health effects of PM on the  
90 progression of diseases mainly depend on gender due to exposure differences, hormonal status and life  
91 stages (Clougherty, 2010). In 2013, PM and outdoor air pollution were classified as carcinogenic to  
92 humans (group 1) by the International Agency of Research on Cancer (International Agency for Research  
93 on Cancer, 2015). Current limit values are defined aiming to lower the concentrations of PM<sub>10</sub> and PM<sub>2.5</sub> in  
94 the air in order to improve air quality. However, ultrafine particles (PM<sub>0.1</sub> or UFPs, *i.e.* particulates of  
95 median aerodynamic diameter  $\leq 0.10 \mu\text{m}$ ) are still under no regulation despite their greater chemical  
96 reactivity and potentially hazardous characteristics. Indeed, they are thought to be more toxic than larger  
97 particles due to their greater number, higher surface area per unit of mass and smaller size allowing them  
98 to reach the deepest areas in lungs and enter the bloodstream resulting in their distribution to other organs

99 (Nemmar *et al.*, 2013; Peters *et al.*, 2006). Deleterious effects of PM involve different mechanisms of toxic  
100 action connected to each other. Firstly, PM can generate reactive oxygen species (ROS) and some  
101 oxidative metabolites, causing oxidative stress. It also induces inflammatory injuries, damages DNA and  
102 causes epigenetic changes (Cho *et al.*, 2018; Kim *et al.*, 2017). Although many studies have been  
103 conducted in recent years, PM toxicity mechanism is not yet clearly understood.

104

105 In this context, following our previous work on the *in vitro* genotoxicity of PM<sub>2.5</sub> from an urban surrounding  
106 (Leclercq *et al.*, 2017), the aim of our work was to investigate the *in vitro* and *in vivo* genotoxic and  
107 mutagenic potential of air pollution FPs (2.5 µm - 0.18 µm) and quasi UFPs (≤ 0.18 µm) collected in the  
108 industrial-port area of Dunkirk (Northern France), near the motorways and the conurbation. For a better  
109 human health assessment, we strove to be as close as possible to the actual *in vivo* exposure conditions  
110 in terms of route of exposure. For that, the collected particles were directly tested on cells or administered  
111 to animals, rather than testing the extracted organic matter (EOM) of the PM, as frequently reported in the  
112 literature for genotoxicity assessment (Abbas *et al.*, 2019; Bełcik *et al.*, 2018; Billet *et al.*, 2018; Bocchi *et al.*,  
113 *et al.*, 2017, 2016; Borgie *et al.*, 2015; Cassoni *et al.*, 2004; Gábelová *et al.*, 2007a, 2007b, 2004; Lemos *et al.*,  
114 *et al.*, 2016; Oh *et al.*, 2011; Topinka *et al.*, 2000). *In vitro* assessment was performed with two human lung  
115 cell models, common immortalized cell lines (BEAS-2B and NCI-H292 cells) and primary cells [normal  
116 human bronchial epithelial cells (NHBE)], by using the *in vitro* comet and micronucleus assays at the dose  
117 of 5 µg PM/cm<sup>2</sup>. These tests have been chosen for their complementarities for the detection of primary  
118 DNA damage and chromosomal aberrations, respectively. These two methodologies appear to be well  
119 adapted to highlight a broad spectrum of genetic and mutagenic events, particularly of clastogenic and  
120 aneugenic types. It is known that after nanoparticles exposure, the comet assay is the most common test  
121 for DNA damage detection, and that the micronucleus test is less time-consuming and thus more used for  
122 nanoparticles testing than the chromosomal aberration assay (Dusinska *et al.*, 2017). Moreover, they  
123 represent the most commonly used combination of tests for nanoparticles genotoxicity studies  
124 (Magdolenova *et al.*, 2014). From a methodological point of view, the comet assay was performed on cells  
125 exposed at the air-liquid interface (ALI) in order to obtain a differentiation state as close as possible to the  
126 cellular exposure conditions observed *in vivo*. ALI cell culture is a useful method for investigating the  
127 harmful effects of PM on the respiratory airway as primary cells exhibit differentiation and pseudostratified  
128 striation similar to those observed *in vivo* (Cho *et al.*, 2018). On the other hand, the micronucleus test

129 requires at least 1.5 to 2 cell divisions to reveal micronucleus formation (OECDa, 2016). However, our  
130 preliminary assays demonstrated that, cells, once at the ALI, differentiate but no longer divide, and thus,  
131 preventing the induction of the micronuclei (data not shown). The micronucleus test was therefore  
132 performed in submerged conditions, knowing that submerged cell culture is still a common method to  
133 assess adverse effects of PM in cell experiments (Bakand, 2016; Fröhlich and Salar-Behzadi, 2014).  
134 Moreover, each assay was coupled to an assessment of cytotoxicity (trypan blue dye exclusion assay and  
135 calculation of the percentage of relative population doubling).

136

137 For *in vivo* assessment, after intranasal exposures of BALB/c mice to FPs and UFPs, genotoxic effects  
138 were assessed using (i) the *in vivo* comet assay on lung (as a primo exposed organ) and on liver (as a  
139 systemic organ, metabolizer and rich in cells capable of phagocytosis), (ii) the *in vivo* micronucleus test on  
140 bone marrow, and (iii) the *in vivo* Pig-A gene mutation assay on peripheral blood, at doses of 10 and/or  
141 100 µg per animal. For ethical and scientific reasons, these three tests were performed on the same  
142 animals. The comet assay was performed in the frame of the acute and subacute (1 month) exposures.  
143 Indeed, as this test is rather an exposure marker, it is more relevant to perform it after a short period of  
144 treatment. It is less appropriate after a long period during which repair and cytotoxicity events may  
145 underestimate the genotoxic response. The micronucleus and Pig-A gene mutation tests, as markers of  
146 effects, were carried out in the two long treatment regimens, subacute (1 month) and subchronic (3  
147 months) exposures.

148

149

150

## 151 **2. MATERIALS AND METHODS**

152

### 153 **2.1. Chemicals and reagents**

154 Phosphate buffered saline (PBS) was purchased from GIBCO Invitrogen (Cergy-Pontoise, France).  
155 Giemsa reagent, mitomycin C (MMC, CAS No. 50-07-7), methyl methanesulfonate (MMS, CAS No. 66-27-  
156 3), ethyl-nitrosourea (ENU, CAS No. 759-73-9), cyclophosphamide (CPA, CAS No. 6055-19-2), penicillin,  
157 streptomycin, amphotericin B, EDTA, propidium iodide, NaCl, Trypan Blue and bovine serum albumin  
158 (BSA) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Acetic acid was from VWR  
159 (Fontenaysous-bois, France). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Noisy le  
160 Grand, France). Hank's balanced salt solution (HBSS) without phenol red, rat tail collagen I solution and  
161 TryPLE™ Express were obtained from ThermoFisher Scientific (Illkirch, France). RPMI 1640, horse  
162 serum, fetal calf serum, non-essential amino acids and phosphate buffered saline (PBS) were purchased  
163 from GIBCO Invitrogen (Cergy-Pontoise, France). BEGM™ BulletKit™, B-ALI™ BulletKit™, B-ALI™ growth  
164 basal medium, B-ALI™ differentiation basal medium, B-ALI™ inducer, Trypsin Neutralizing Solution were  
165 purchased from Lonza (Verviers, Belgium).

166

### 167 **2.2. Particles sampling**

168 FPs and UFPs were collected at approximately 2 km south of the Dunkirk industrial harbor area (North of  
169 France) with our previously described protocol (Saleh *et al.*, 2019). Briefly, the sampling was conducted  
170 using a High-Volume Impactor Sampler (HVIS, flow rate of 400 L/min) in order to collect the fine fraction  
171 by impaction on Teflon stripes and the UFPs fraction by filtration on 8"x10" polycarbonate (PC)  
172 membranes. The cumulated fine fraction was transferred with a Teflon tip into an acid cleaned dry vial,  
173 weighted and stored along with all the UFPs PC membranes at -20°C until chemical analyses and  
174 toxicological assays.

175

### 176 **2.3. Physicochemical characterization**

177 Trace metals measurements, polycyclic aromatic hydrocarbons (PAHs) analysis, and particles size  
178 distribution were performed in both size fractions and described in our previous study (Saleh *et al.*, 2019).  
179 Briefly, concentrations of trace elements were measured after microwave acid digestion by ICP-MS  
180 (NeXion 300x, Perkin Elmer). For PAHs, analysis was performed after pressurized liquid extraction in



181 acetonitrile with a Dionex ASE 200 instrument (ThermoFisher Scientific) by high-pressure liquid  
182 chromatography consisting of a Waters 2695 Alliance system (Waters SA, Saint-Quentin-en-Yvelines,  
183 France) coupled to an on-line 996-photodiode array and a 2475-fluorimetric detector. The hydrodynamic  
184 diameter of FPs and UFPs was measured after suspending particles in HBSS (1mg/mL) using a Dynamic  
185 Laser Scatter Zetasizer nano ZS (Malvern Instruments, Orsay, France).

186

## 187 **2.4. *In vitro* genotoxicity and cytotoxicity assessment**

188 *In vitro* genotoxicity assays, treatment conditions and cells used in our study are summarized in Table 1.

189

### 190 **2.4.1. Preparation of particles suspensions**

191 Firstly, FP and UFP samples were suspended in HBSS. Then, these initial particles suspensions were  
192 prepared following a protocol described by Jensen *et al.* in the NANOGENOTOX Joint Action (Jensen *et*  
193 *al.*, 2011). Briefly, UFP and FPs were suspended in 0.05% BSA-water in order to obtain a 10x initial  
194 particles solution (BSA-water was prepared by dissolving 0.05% w/v BSA in distilled water and sterile-  
195 filtered) and dispersed by probe-sonication for 16 min, at 400W and 10% amplitude using a 400 Watt  
196 Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA). UFPs and FPs suspensions  
197 were cooled on ice to minimize heat-development during sonication. These initial suspensions were then  
198 diluted 1:9 v/v in cell culture medium, supplemented with amphotericin B (20 mg/mL) at 2% (v/v), at the  
199 time of cell treatment to obtain final suspensions at 5 µg PM/cm<sup>2</sup>. Initial and final suspensions were  
200 carefully vortexed before each pipetting.

201

### 202 **2.4.2. Cell lines and culture conditions**

#### 203 **2.4.2.1. Primary cells: NHBE cells**

204 NHBE cells (Lonza, Verviers, Belgium) were isolated from donated human tissue (from epithelial lining of  
205 airways above bifurcation of the lungs), after obtaining permission for their use in research applications by  
206 informed consent or legal authorization. The characteristics of the healthy donor and cells are shown in  
207 Table 2. Cells were cultured in 75 cm<sup>2</sup> Corning® CellBIND® surface cell culture flasks (Sigma-Aldrich, St.  
208 Quentin Fallavier, France) in bronchial epithelial growth medium BEGM according to the manufacturer  
209 recommendations. Medium was renewed every 3 days and cells were subcultured once a week and  
210 seeded at a density of 5x10<sup>5</sup> cells per flask. The doubling time of NHBE cells was approximately 30 hours

211 (this may vary depending on the donor). Their characteristics were described recently by Rayner *et al.*  
212 (2019).

213

#### 214 **2.4.2.2. Immortalised cells: BEAS-2B and NCI-H292 cells**

215 **NCI-H292 cells.** The human muco-epidermoid bronchiolar carcinoma cell line NCI-H292 was obtained  
216 from the American Type Culture Collection (ATCC<sup>®</sup> CRL-1848<sup>™</sup>). Cells were seeded at a density of  $5 \times 10^5$   
217 cells per culture flask (75 cm<sup>2</sup>; Falcon<sup>™</sup>, Becton Dickinson Biosciences, France) and maintained at 37°C  
218 in a humidified 5% CO<sub>2</sub> atmosphere in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf  
219 serum, 200 UI/mL penicillin, 50 mg/L streptomycin, 2.5 mg/L amphotericin B. This medium is hereafter  
220 referred to as RPMI 10 growth medium. Medium was renewed every 2 to 3 days and cells were  
221 subcultured when they reached 80-90 % confluence. The ATCC indicates that the doubling time of NCI-  
222 H292 cells is approximately 48 hours.

223

224 **BEAS-2B cells.** The human bronchial epithelial cell line BEAS-2B was obtained from the American Type  
225 Culture Collection (ATCC<sup>®</sup> CRL-9609<sup>™</sup>). Comparable to the NHBE cells, BEAS-2B cells were seeded at a  
226 density of  $5 \times 10^5$  cells in 75 cm<sup>2</sup> Corning<sup>®</sup> CellBIND<sup>®</sup> surface cell culture flasks and maintained at 37°C in a  
227 humidified 5% CO<sub>2</sub> atmosphere in BEGM. Medium was renewed every 2 to 3 days and cells were  
228 subcultured before reaching confluence since confluent cultures rapidly undergo squamous terminal  
229 differentiation. The doubling time of BEAS-2B cells is approximately 24 hours (Costa *et al.*, 2010; Haniu *et*  
230 *al.*, 2013).

231

#### 232 **2.4.3. In vitro comet assay**

##### 233 **2.4.3.1. Cell treatment at the air / liquid interface (ALI)**

234 Cells were seeded at a density of  $2.5 \times 10^5$  cells/insert for NHBE cells or at a density of  $8 \times 10^4$  cells/insert  
235 for BEAS-2B and NCI-H292 cells, on 24 mm Transwell<sup>®</sup> inserts (with 0.4 µm pore polyester membrane,  
236 thickness of 10 µm) in a 6-well microplate (Corning<sup>®</sup> Transwell<sup>®</sup> polyester membrane cell culture inserts,  
237 Sigma Aldrich, Saint-Quentin Fallavier, France) pre-coated with 0.03 mg/mL rat tail collagen I solution.  
238 Cells were grown submerged in medium until reaching 90-100% confluency, *i.e.* ~ 5-7 days for NHBE  
239 cells in B-ALI<sup>™</sup> growth medium according to the manufacturer recommendations, ~3 days in BEGM for  
240 BEAS-2B cells and ~3 days in RPMI 10 for NCI-H292 cells.. For NBHE cells, B-ALI<sup>™</sup> growth medium was

241 replaced by B-ALI™ differentiation medium with 0.2% (v/v) B-ALI™ inducer. For NHBE cells, differentiation  
242 at the ALI is reached after 21 days of culture. This effective differentiation was already checked by  
243 Leclercq *et al.* (2016) and Sotty *et al.* (2019). One experiment was performed with duplicate cultures (*i.e.* 2  
244 biological replicates) for each group (*i.e.* 2 wells per dose). Each treatment (without metabolic activation)  
245 was coupled to an assessment of cytotoxicity as described below. In each insert, the apical medium was  
246 removed to expose the cells. Particles suspensions (as described in § 2.4.1) were then directly deposited  
247 by pipetting on the permeable membrane inserts (300 µL/insert), for 4 h without recovery time (4h+0h).  
248 Then cells were washed twice with PBS and immediately harvested by trypsinization with 1 mL/well of  
249 trypsin-EDTA for NHBE cells or 1 mL/well of TrypLE™ Express for BEAS-2B and NCI-H292 cells, for 4 min  
250 at 37°C. Then, 1 mL/well of Trypsin Neutralizing Solution for NHBE cells, 1 mL/well of RPMI 10 growth  
251 medium for NCI-H292 cells, or 1 mL/well of BEGM for BEAS-2B cells, was added in order to stop the  
252 action of trypsin. Cells were transferred to 15 mL polypropylene tubes and centrifuged at 1000 rpm for 5  
253 min. Supernatant was discarded and the pelleted cells were immediately used for the comet assay.  
254 Positive control was MMS (0.96 µg/cm<sup>2</sup>) and negative control was culture medium.

255

#### 256 **2.4.3.2. Comet assay protocol**

257 The comet assay was performed under alkaline conditions (pH>13) in compliance with previously  
258 described protocol (Singh *et al.*, 1988; Tice *et al.*, 2000; Witte *et al.*, 2007). For each group 2 duplicate  
259 slides per culture were prepared (*i.e.* 4 technical replicates in total per concentration). When scoring for  
260 DNA migration, slides were independently coded and analyzed after addition of 25 µL of propidium iodide  
261 (20 µg/mL in distilled water) and a coverslip. Slides were then examined at 250x magnification using a  
262 fluorescence microscope (Leica Microsystems SAS - DM 2000, Heerbrugg, Switzerland) equipped with an  
263 excitation filter of 515-560 nm and a 590 nm barrier filter, connected through a gated monochrome CCD  
264 IEEE1394 FireWire video camera (Allied Vision Technologies) to the Comet Assay IV Image Analysis  
265 System, version 4.11 (Perceptive Instruments Ltd, Suffolk, United Kingdom). Four hundred randomly  
266 selected cells per concentration (100 cells from each of the 2 duplicate slides in the 2 cultures) were  
267 scored. DNA damage was expressed as percentage of DNA in the tail (% tail intensity) (Burlinson *et al.*,  
268 2007; Lovell and Omori, 2008).

269

#### 270 **2.4.3.3. Cytotoxicity assessment**

271 For the comet assay, cytotoxicity was assessed at harvest using the Trypan Blue dye exclusion assay.  
272 Concentrations which led to more than 70% of viability were then submitted to a genotoxicity assessment.

273

#### 274 **2.4.4. *In vitro* micronucleus test**

##### 275 **2.4.4.1. *Cell treatment in submerged conditions***

276 NHBE and BEAS-2B cells were seeded at a density of  $1.5 \times 10^5$  cells/well in BEGM in 6-well Corning®  
277 CellBIND® plates (Sigma-Aldrich, Saint-Quentin Fallavier, France). NCI-H292 cells were seeded at a  
278 density of  $3 \times 10^5$  cells/well in RPMI 10 in 6-well cell culture plates. In order to allow exponential cell growth  
279 and maximum cell attachment, cells were incubated for 24 h (NHBE cells) or 48 h (BEAS-2B and NCI-  
280 H292 cells) prior to treatment. One experiment was performed with duplicate cultures (*i.e.* 2 wells per  
281 dose). Each treatment (without metabolic activation) was coupled to an assessment of cytotoxicity as  
282 described below. Cells were treated (2 mL/well) for 24 h then washed twice with PBS and fresh medium  
283 was added. Plates were incubated at 37°C for an additional 5 days recovery period (24h/+120h) for NHBE  
284 cells or 2 days recovery period (24h/+48h) for BEAS-2B and NCI-H292 cells. At the end of the recovery  
285 time, the harvest was the same as for the comet assay (see 2.4.3.1). Positive control was MMC (0.01  
286  $\mu\text{g}/\text{cm}^2$ ) and negative control was culture medium.

287

##### 288 **2.4.4.2. *Micronucleus test protocol***

289 At the harvest time, pelleted cells were treated with a hypotonic solution (BEGM diluted 1:3 v/v in distilled  
290 water, 2 mL/tube, 10 min for NHBE cells; BEGM diluted 1:3 v/v in distilled water, 2 mL/tube, 8 min for  
291 BEAS-2B cells; RPMI 10 diluted 1:1 v/v in distilled water, 5 mL/tube, 7.5 min for NCI-H292 cells). At the  
292 end of the hypotonic shock, a pre-fixation step was performed by adding very slowly 500  $\mu\text{L}/\text{tube}$  of 5% (in  
293 medium) cold Carnoy mixture (ethanol/acetic acid, 3:1 v/v). Cells were then centrifuged at 1000 rpm for 5  
294 min and pelleted cells were fixed for at least 15 min with 2 mL/tube of pure cold Carnoy mixture (this  
295 fixation step was performed twice for NHBE cells). After another centrifugation, cells were spread on glass  
296 slides, air-dried at room temperature, stained for 10 min with 2% (NHBE cells) or 4% (BEAS-2B and NCI-  
297 H292 cells) Giemsa mineral water solution, rinsed with distilled water and finally dried at room  
298 temperature. For each group, 2 duplicate slides per culture were prepared for analysis (*i.e.* 4 technical  
299 replicates in total per concentration). Slides were independently coded and analyzed. Micronuclei (MN),

300 identified according to recommended criteria (Fenech, 2000; Fenech *et al.*, 2003), were scored in 1000  
301 intact mononucleated cells per slide (4000 mononucleated cells/concentration) at 500x magnification.

#### 302 **2.4.4.3. Cytotoxicity assessment**

303 For the micronucleus test, measurements of cell proliferation are made to assure that sufficient treated  
304 cells have undergone mitosis during the test. For this, the percentage of Relative Population Doubling  
305 (RPD) as recommended by OECD test guideline No. 487 (OECD, 2016a) was calculated:

306  $RPD = [(Number\ of\ Population\ doublings\ in\ treated\ cultures) \div (Number\ of\ Population\ doublings\ in\ control$   
307  $cultures)] \times 100$  where Population Doubling =  $[\log (Post\text{-}treatment\ cell\ number \div Initial\ cell\ number)] \div \log$   
308 2.

309 Concentrations leading to a RPD greater than 45±5% were considered as being of low or no cytotoxicity  
310 and were then submitted to genotoxicity assessment.

311

### 312 **2.5. *In vivo* genotoxicity assessment**

313 The genotoxic/mutagenic potential of FPs and UFPs was analyzed by a battery of three *in vivo* tests  
314 composed of the comet assay, the micronucleus assay and the Pig-A gene mutation assay. Tests, end-  
315 points, target organs and treatment schedules are summarized in Table 3.

316

#### 317 **2.5.1. Animal model**

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

323

#### 324 **2.5.2. Treatments**

325 Three exposure protocols were realized in this study: mice were exposed twice at 24-hour intervals (acute  
326 exposure) or three times/week during 1 and 3 months (subchronic exposures). Animals were anesthetized  
327 with 2% isoflurane and exposed to FPs or UFPs by intranasal instillation at the selected dose of 100 µg for  
328 acute exposure and 10 µg for subchronic exposures. Particles were suspended in 37 µL of sterile HBSS  
329 (*i.e.* approximately 2 mL/kg). A negative control group received intranasally 37 µL of HBSS and 3 positive

330 control groups were treated (1) orally with MMS [100 mg/kg b.w/day (x2) in sterile water] for the comet  
331 assay (combined or not to the micronucleus test), (2) intraperitoneally with CPA [50 mg/ kg b.w/day (x1) in  
332 0,9 % NaCl] for the micronucleus test, or (3) ENU [40 mg/ kg b.w/day (x3) in sterile water] for the Pig-A  
333 assay. Animal body weights were recorded before each administration while clinical signs were monitored  
334 daily (data not shown).

335

### 336 **2.5.3. *In vivo* comet assay**

337 The potential genotoxic activity of FPs and UFPs was tested using the *in vivo* comet assay in isolated lung  
338 and liver cells in compliance with the OECD test guideline No. 489 (OECD, 2016b), using 2 successive  
339 administrations at 24-hour intervals (see 2.5.2), followed by one sampling time 2-6 hours after the last  
340 treatment. At the sampling time, treated animals were anaesthetized with 10 µL/g of a mix of ketamine  
341 (100 mg/kg) / xylazine (20 mg/kg) administered intraperitoneally, and exsanguined. 'V' shaped incision  
342 was made from the center of the lower abdomen to the rib cage. The skin and muscles were removed to  
343 reveal the abdominal cavity. A portion of the lung and liver were removed and washed in the cold fresh  
344 mincing buffer (10% v/v EDTA 200 mM, 80% v/v HBSS without phenol red and 10% v/v DMSO; pH 7.5)  
345 until as much blood as possible has been removed. The portion was minced with a pair of fine scissors to  
346 release the cells. Cell suspension was stored on ice for 15-30 seconds to allow large clumps to settle. The  
347 whole cell suspension was collected. Cells were enumerated on a haemocytometer and  $2 \times 10^4$  viable  
348 cells per slide were sampled from each cell suspension for proceeding to slide preparation,  
349 electrophoresis and scoring (as described previously for the *in vitro* comet assay. See 2.4.3.2). Three  
350 slides were prepared per animal (5 animals per group). After coding slides, 750 randomly selected cells  
351 per group (*i.e.* 50 cells per slide, 3 slides per animal, 5 animals per group), were analysed.

352

### 353 **2.5.4. *In vivo* micronucleus test**

354 The potential genotoxic activity of FPs and UFPs was tested using the *in vivo* micronucleus test in the  
355 bone marrow of treated mice during 1 and 3 months, in compliance with the OECD test guideline No. 474  
356 (OECD, 2016c). At the sampling time, animals were sacrificed (5 animals per group). The femurs were  
357 removed and the bone marrow was extracted with 1 mL of fetal calf serum. The supernatant was removed  
358 and after homogenisation, the centrifugate was spread on slides (2 slides per animal). The smears were  
359 stained using a technique derived from the May Grunwald Giemsa technique (Schmid, 1975), which

360 makes it possible to distinguish between immature and mature erythrocytes, *i.e.* polychromatic (PCE) and  
361 normochromatic erythrocytes (NCE), respectively. PCE are purple whereas NCE are red. After coding  
362 slides, the number of PCE having one or more micronuclei (micronucleated polychromatic erythrocytes,  
363 MNPCE) was determined from the microscopic examination of at least 4000 PCE per animal (*i.e.* 2000  
364 PCE per slide). The PCE/NCE ratio was determined by analyzing at least 1000 erythrocytes per animal  
365 (*i.e.* 500 erythrocytes per slide).

366

#### 367 **2.5.5. *In vivo* Pig-A gene mutation assay**

368 The purpose of the *in vivo* erythrocytes-based *Pig-A* gene mutation assay is to quantify *in vivo* mutation  
369 through a flow cytometry-based approach in somatic cells of treated animals (Bryce *et al.*, 2008;  
370 Dobrovolsky *et al.*, 2010; Kimoto *et al.*, 2011). Blood samples were collected at the end of each chronic  
371 exposure, *i.e.* on the 28<sup>th</sup> day and the 45<sup>th</sup> day, by retro-orbital bleeding after isoflurane anaesthesia.  
372 Approximately 120 µL of blood were collected into heparinized capillary tubes (Fisher Scientific).  
373 Immediately upon collection, 60 µL exactly of each blood sample were transferred into tubes containing  
374 100 µL kit-supplied heparin solution where they remained at room temperature for less than 2 hours until  
375 sample preparation according to supplier's protocol (MutaFlowPLUS Kits Mouse Blood (Litron, Rochester,  
376 New York). A FACSCanto II flow cytometer (BD Biosciences) providing 488 nm and 633 nm excitation,  
377 running FACSDiva™ v7.0 software, was used for data acquisition and analysis. Frequencies of mutant  
378 reticulocytes (RET) and mutant red blood cells (RBC) were determined by examining at least  $1 \times 10^6$  of  
379 total RET or RBC, respectively, per animal. All mutant cell frequencies, %RET, averages and standard  
380 error calculations were performed with the Excel® spreadsheet developed by the supplier (Litron).

381

#### 382 **2.6. Statistical Analysis**

383 For each test, differences with a  $p < 0.05$  were considered statistically significant.

384 For the *in vitro* and *in vivo* comet assay, it has previously been shown that the tail intensity does not follow  
385 a Gaussian distribution (Bauer *et al.*, 1998). Thus, the non-parametric Mann-Whitney U-test was used to  
386 evaluate the statistical difference between groups (*i.e.* between each concentration vs. the respective  
387 negative control). All statistical analyses were performed with StatView® Software (version 5.0, SAS  
388 Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, USA).

389 For the *in vitro* micronucleus test, the statistical significance of difference between groups (*i.e.* each  
390 concentration vs. the negative control) was determined using the CHI-squared test.

391 For the *in vivo* micronucleus test, statistical analyses were performed for micronucleus number with  
392 StatView<sup>®</sup> Software using the non-parametric Mann-Whitney U-test. The statistical comparison for the  
393 PCE/NCE ratio was performed using the Student's t test.

394 For the *in vivo* Pig-A gene mutation assay, a pair-wise comparison using the Dunnet's t-test was  
395 performed.



## 396 **3. RESULTS**

397

### 398 **3.1. *In vitro* genotoxicity and cytotoxicity assessment**

399 Results of the *in vitro* tests are summarized in Table 4.

400

#### 401 **3.1.1. *In vitro* comet assay**

402 Primary DNA damage was measured with the *in vitro* comet assay after a 4 h treatment. Results of the  
403 means of medians of percentage of tail intensity (TI) for each group and the corresponding relative cell  
404 viability obtained in BEAS-2B, NCI-H292 and NHBE cells are presented in Figure 1. To exclude  
405 cytotoxicity as a confounding factor, the cell viability should be more than 70 % for the comet assay  
406 (Collins *et al.*, 2008; Tice *et al.*, 2000; Witte *et al.*, 2007). Figure 1 shows that both types of airborne  
407 particles did not induce any significant cytotoxic effects at the tested concentration of 5 µg PM/cm<sup>2</sup>, as  
408 assessed by the trypan blue dye exclusion assay. Moreover, under tested conditions, no increase in DNA  
409 strand breaks was observed in NHBE cells, whatever the type of particles. On the contrary, slight but  
410 statistically significant increases (p<0.05) in TI were obtained in BEAS-2B cells (6.55 % and 4.19 % for  
411 FPS and UFPs, respectively, vs. 1.54 % for the negative control). For NCI-H292 cells, increases in TI  
412 were observed for both particles, statistically significant only for FPs [12.24% for FPs (p<0.05) and 10.25%  
413 for UFPs, vs. 4.97 % for the negative control].

414

#### 415 **3.1.2. *In vitro* micronucleus test**

416 The *in vitro* micronucleus test was used to assess the induction of chromosomal aberrations  
417 (clastogenicity and/or aneuploidy) after a 24 h treatment.. Micronuclei frequencies and the corresponding  
418 cytotoxicity (% RPD) are shown in Figure 2. No cytotoxic effect was observed, except for UFPs on NCI-  
419 H292 for which cell viability was only 30.50 %. For both airborne particles tested, no genotoxic activity  
420 were noted since no statistically or biologically significant effect was observed, in any of the three cell lines  
421 (even with UFPs in NCI-H292 cells despite a high level of toxicity noticed).

422

### 423 **3.2. *In vivo* genotoxicity assessment**

424 Results of the *in vivo* tests are summarized in Table 5.

425

426 **3.2.1. Acute exposure**

427 The genotoxic potential of the airborne particles was investigated in the *in vivo* comet assay, on isolated  
428 lung and liver cells of mice after an acute exposure (2 treatments at 24-hour intervals at the highest dose  
429 of 100 µg of particles per animal). Results for the mean of medians of percentage of TI are given in Figure  
430 3. For FPs and UFPs, very low levels of TI (< 2.5 %), close to those obtained for the negative controls,  
431 were observed in the two selected organs.

432

433 **3.2.2. Subacute exposure**

434 Results of genotoxicity/mutagenicity assessment after the subacute exposure of mice (1 month, 10 µg of  
435 particles per animal, 3 times a week) are presented in Figure 4.

436 For FPs and UFPs, no statistically significant increase in the level of DNA damage was observed (TI < 1.2  
437 %), in either the liver or the lungs (Figure 4A and 4B).

438 Regarding the frequency of MNPCE, no significant increase was found in animals treated with FPs or  
439 UFPs (~ 1 ‰) when compared to the control group (0.6 ‰) (Figure 4C).. Airborne particles treatment did  
440 not significantly affect the ratio PCE/NCE indicating the absence of cytotoxic effects on the bone marrow  
441 at this dose level.

442 The frequencies of mutants RET (<  $1.6 \times 10^{-6}$  cells) and mutants RBC (<  $0.7 \times 10^{-6}$  cells) did not shown  
443 any statistically significant increase in the animals treated with FPs or UFPs when compared to the control  
444 group (RET =  $0.6 \times 10^{-6}$  cells and RBC =  $0.9 \times 10^{-6}$  cells) (Figure 4D). The percentage of RET is the ratio  
445 of newly formed RNA-positive erythrocyte relative to all erythrocytes, and is used as a measure of bone  
446 marrow cytotoxicity. FPs or UFPs treated mice did not exhibit any significant changes in % RET at day 28,  
447 thus confirming the absence of toxicity.

448

449 **3.2.3. Subchronic exposure**

450 Summarized results obtained after the subchronic exposure of animals (3 months, 10 µg of particles per  
451 animal, 3 times a week) are presented in Figure 5.

452 No induction of MN formation was observed in mice treated with FPs or UFPs (< 0.7 ‰). No decrease of  
453 the ratio PCE/NCE was observed (Figure 5A).

454 No statistically significant increase in mutant frequencies of RBC and RET (<  $3 \times 10^{-6}$  cells) was observed  
455 for the UFPs compared to the negative control (RET =  $0.5 \times 10^{-6}$  cells and RBC =  $0.7 \times 10^{-6}$  cells) (Figure

456 5B). For FPs, a slight increase in frequencies of mutants RBC and mutants RET ( $< 22 \times 10^{-6}$ ) was found,  
457 but it remained non-statistically significant compared to the negative control. The absence of toxicity on  
458 the bone marrow was confirmed (only very slight decreases, non-statistically significant, in the % RET).

459

460

#### 461 4. DISCUSSION

462 Respiratory diseases are one of the leading causes of death in the world and constitute a major challenge  
463 in terms of public health (Ferkol and Schraufnagel, 2014; Schraufnagel *et al.*, 2019). The prevalence of  
464 these diseases is constantly increasing in the Hauts-de-France region (northern France), where the tested  
465 particles were collected (Agence Régionale de Santé des Hauts-de-France, 2017; Lapôte-Ledoux *et al.*,  
466 2019). Numerous epidemiological studies have highlighted the role of air quality in respiratory morbidity  
467 and mortality (Amini *et al.*, 2019; Nabizadeh *et al.*, 2019; Shamsipour *et al.*, 2019; Weber *et al.*, 2016; Xing  
468 *et al.*, 2016; Yitshak-Sade *et al.*, 2019). Anthropogenic air pollution appears to be responsible for  
469 increasing the incidence and aggravation of diseases such as asthma, allergies or COPD (Guerreiro *et al.*,  
470 2018; Xia *et al.*, 2016). Although air pollution is now a well-suspected risk factor of lung cancer (Loomis *et al.*,  
471 2013), to our knowledge no work has been conducted to specifically study *in vitro* and *in vivo*  
472 genotoxic and mutagenic effects induced by FPs and UFPs. To fill this gap, assays were implemented to  
473 better determine whether genetic/mutagenic endpoints, in *in vitro* lung cell models and in an animal model,  
474 would be impacted after exposure to atmospheric FPs and UFPs collected in an area characterized by  
475 intense industrial activity and heavy high traffic roads.

476  
477 For the *in vitro* approach, we used two types of lung cell models: standard cell lines (BEAS-2B and NCI-  
478 H292 cells) and human primary cells (NHBE cells). In order to be as close as possible to the “real” *in vivo*  
479 exposure conditions, the comet assay was performed on cells exposed at the ALI. Because of the  
480 absolute need of proliferative cells for the study of chromosomal aberrations, the *in vitro* micronucleus test  
481 cells were performed on cells grown in submerged conditions (under these conditions, cells could not be  
482 differentiated). The choice of the PM concentration for cell exposure was based on preliminary cell viability  
483 assays (measures of the amount of intracellular ATP and release of G6PD in the culture medium). The  
484 concentration of 5 µg PM/cm<sup>2</sup> corresponds to the 10% lethal concentration (LC<sub>10</sub>) (Sotty *et al.*, 2019). This  
485 was confirmed by our data, since, in our test conditions, FPs and UFPs did not induce any significant  
486 cytotoxic effects, neither in the comet assay nor in the micronucleus test, in the three cell types (except for  
487 UFPs which induced *ca.* 70% of cytotoxicity on NCI-H292 in the micronucleus test). We have shown that  
488 for primary NHBE cells, negative results were obtained for both endpoints, as observed *in vivo*. As  
489 expected, primary cells appeared to be more predictive of an *in vivo* effect. The lack of genotoxic activity  
490 observed with NHBE cells was also consistent with our previously published data on the *in vitro*

491 genotoxicity of air pollution-derived PM<sub>2.5</sub> collected in Lille (northern France) during two different seasons  
492 (*i.e.* autumn-winter and spring-summer) (Leclercq *et al.*, 2017). Otherwise, immortalized BEAS-2B and  
493 NCI-H292 cells induced a positive response in the comet assay but no effect was noticed in the  
494 micronucleus test. In these cell lines, the observed primary DNA damage did not lead to chromosomal  
495 aberrations suggesting a potential involvement of DNA repair processes. BEAS-2B and NCI-H292 are  
496 both well characterized P53 proficient cells. In terms of metabolic capacity, NCI-H292 and BEAS-2B cell  
497 lines exhibited high homology in gene expression pattern with primary cells (xenobiotic metabolizing  
498 enzymes, transporters and nuclear receptors) (Courcot *et al.*, 2012; Garcia-Canton *et al.*, 2013). Thus,  
499 they could be used as surrogates for investigating the metabolism and bioactivation of toxicants in  
500 bronchial epithelium. However, we can hypothesize that their origin (cancerous cells for NCI-H292 and 12-  
501 SV40 hybrid virus transformed cells for BEAS-2B) and their poor detoxification capabilities (especially in  
502 antioxidant systems) make them more sensitive to primary DNA damage. Indeed, as stated by Bhowmick  
503 and Gappa-Fahlenkamp (2016), when NCI-H292 cells are cultured at ALI and exposed to cigarette  
504 smoke, they suffer considerable DNA damage. Moreover, it is known that BEAS-2B and NCI-H292 cells  
505 were relatively inefficient regarding the formation of tight intercellular junctions.

506 On the other hand, it is well known that chemical composition of the particles plays an important role in  
507 induction of toxicity. Despite the occurrence of PAHs (benzo[a]pyrene, indenopyrene, fluoranthene,  
508 benzo[b]fluoranthene...) and metals (As, Ni, Pb, V, Cd, Cu, Zn, Fe, Cr...) in our FPs and UFPs samples  
509 (Saleh *et al.*, 2019), some of which being classified by IARC as carcinogenic, no genotoxicity was  
510 observed in NHBE cells. Our results, obtained with native particles samples, may seem at odds with  
511 numerous studies conducted mainly almost exclusively on PM extractable organic matter (PM-EOM)  
512 (Abbas *et al.*, 2019; Belcik *et al.*, 2018; Billet *et al.*, 2018; Bocchi *et al.*, 2017; Gábelová *et al.*, 2004;  
513 Lemos *et al.*, 2016; Marabini *et al.*, 2017, 2017; Pereira *et al.*, 2013). We can thus hypothesize that  
514 chemicals adsorbed on particles, in particular PAHs, nitro-PAHs and other related compounds (dioxins  
515 and furans), were probably not enough bioavailable, or in too small amounts compared to the sensitivity of  
516 test systems, to exert their genotoxic effect. However, as explained by André *et al.* (2011), extrapolations  
517 of results obtained with organic extracts to potential effects on human health are limited for three main  
518 reasons: (i) there are too high chemical concentrations in the PM-EOM compared to environmental  
519 concentrations; (ii) PM-EOM does not exactly match what is found in “real life”, *i.e.* in the whole complex  
520 mixture-coated onto the particles (especially metals not extractable by organic solvents); (iii) toxicity of the

521 core of the particles is not assessed. Nevertheless, increases in DNA damage and in chromosomal  
522 aberrations were not systematically reported since some negative genotoxicity results were also found in  
523 the literature. For example, André *et al.* (2011) have directly tested suspensions of PM<sub>2.5</sub> collected in  
524 Dunkirk. Exposure of A549 immortalized pulmonary cells to PM<sub>2.5</sub> for 72 h at the LC50 (31.6 µg PM/cm<sup>2</sup>)  
525 did not lead to quantifiable PAH bulky DNA adducts (although A549 cells are cancerous cells with highly  
526 active phase I metabolism enzymes). Recently, Billet *et al.* (2018) observed neither the formation of DNA  
527 bulky adducts after a 72 h-exposure of BEAS-2B cells to PM<sub>0.3-2.5</sub> or to PM-EOM, despite the high  
528 sensitivity of the <sup>32</sup>P post-labeling used method. Abbas noticed slight but significant decreases of  
529 phosphorylated-Histone 2AX (γH2AX) in BEAS-2B cells 72 h after their exposure to the organic fraction of  
530 air pollution-derived PM<sub>2.5</sub> from 3 to 30 µg PM<sub>2.5</sub>-eq/cm<sup>2</sup> (Abbas *et al.*, 2019). In return, 8-OHdG adducts  
531 were measured as significantly increased after 48 h of treatment but exclusively at the highest  
532 concentration of 15 µg PM/cm<sup>2</sup> (not at 3 µg PM/cm<sup>2</sup>). However, 8-OHdG formation was shown in A549  
533 cells after incubation for 24 h, 48 h and 72 h with whole PM<sub>2.5</sub> from Dunkirk at concentrations  
534 corresponding to the LC10 (6.33 µg PM/cm<sup>2</sup>) and LC50 (31.6 µg PM/cm<sup>2</sup>) (André *et al.*, 2011). Lepers *et al.*  
535 *et al.* (2014) also observed positive responses in the comet and in the micronucleus assays, as well as for  
536 histone H2AX phosphorylation, in the BEAS-2B cell line exposed for 72 h to native PM samples from  
537 industrial, urban, and rural areas of Dunkirk, and exclusively at the higher concentration tested of 15 µg  
538 PM/cm<sup>2</sup> (no genotoxic effect appeared at the lower dose of 3.75 µg PM/cm<sup>2</sup>). This suggests that, in our  
539 study, the concentration (5 µg PM/cm<sup>2</sup>) and treatment times (4 h for the comet assay and 24 h for the  
540 micronucleus test), although more realistic, were probably not high and not long enough, respectively, to  
541 provide measurable genotoxic effects in all cellular models and in all end-points because of their  
542 sensitivity. However, we are aware that the PM concentrations we used in our *in vitro* study are quite far  
543 from environmental exposure expected in humans. Recently, Gualtieri *et al.* (2018) studied the effects of  
544 air pollution on BEAS-2B cells exposed at the ALI under 'real' environmental conditions. They showed that  
545 the total theoretically deposited mass, being in the order of 10<sup>-2</sup> ng/cm<sup>2</sup> is roughly 3 orders of magnitude  
546 lower than the doses usually reported in PM *in vitro* toxicological studies performed under laboratory  
547 conditions (Gualtieri *et al.*, 2018).

548 In summary, for the *in vitro* approach, various results have been reported in the literature proving the  
549 complexity of the study of the genotoxic response after exposure to a heterogeneous mixture represented  
550 by PM. Our work suggests the need to develop appropriate and standard methodologies for assessing the

551 *in vitro* genotoxicity of PM providing more reliable and reproducible data. Few years ago, as stated by  
552 Magdalenova *et al.* (2014), although the number of studies on the genotoxicity of engineered NPs was  
553 increasing, many conflicting results were published. However, conditions influencing the results of NP  
554 genotoxicity studies are now well known (Magdolenova *et al.*, 2014) and recommendations on method  
555 standardization and assay modifications have been made. The same observation can be done for  
556 airborne PM since a diversity of genotoxicity assays and test systems has been used leading to a  
557 multiplicity of results. Thus, it is difficult to compare results without standardized methods. Variability in the  
558 results of genotoxicity studies can be attributed to the emission sources of PM (natural vs. anthropogenic,  
559 rural vs. industrial, heavy fuel combustion vs. road traffic...) conditioning their physicochemical  
560 characteristics (size, solubility, aggregation/agglomeration, composition, presence of mutagens and  
561 transition metals...), and to the experimental conditions. This last point is of major importance when  
562 comparing studies. Indeed, several factors in *in vitro* experimental conditions could influence the results of  
563 genotoxicity testing, such as types of PM extraction (aqueous media, organic solvent, thermal  
564 desorption...) and thus types of particles tested (fractions more or less polar, or native PM), preparation of  
565 PM suspensions (solvents and methods used for suspending and dispersing them), cellular models used  
566 [primary vs. immortalized cultures, normal vs. cancerous cells, human vs. rodent origin, p53 proficient vs.  
567 p53 deficient, high inter-individual variability of the cell donors (Sotty *et al.*, 2019), variation in metabolic  
568 activities, antioxidant and DNA repair capabilities, internalization/phagocytosis properties...], methods of  
569 exposure (ALI vs. submerged conditions), treatment times (short vs. long, acute vs. repeated), doses (low  
570 and realistic vs. high), and cytotoxicity measurements. In addition, it is important to avoid interference of  
571 PM with the genotoxicity assay and to include sufficient controls to avoid false positive/negative results.

572  
573 For the *in vivo* approach, animal models, especially rodents, have already been the subject of many  
574 studies to assess health effects of airborne PM<sub>2.5</sub> such as respiratory diseases, cardiovascular  
575 dysfunctions, diabetes mellitus and allergic sensitization (Cho *et al.*, 2018). However, no data has been  
576 found in the literature regarding the *in vivo* genotoxicity assessment of PM<sub>2.5</sub> and PM<sub>0.1</sub> administered by  
577 the pulmonary route, which highlights the innovative nature of our work. Indeed, we performed a complete  
578 study of the genotoxic potential of FPs and UFPs both in terms of genotoxic endpoints studied (primary  
579 DNA damage, chromosomal aberrations, gene mutations) and duration of treatment performed (acute,  
580 subacute and subchronic). Animals were exposed to particles at the doses of 100 µg and 10 µg for acute

581 and subchronic exposures, respectively. We have estimated that intratracheal instillation of 10  $\mu\text{g}$  of  $\text{PM}_{2.5}$   
582 to BALB/c mice [whose minute ventilation is 57 mL/min according to de Hennezel *et al.* (2001)] is  
583 equivalent to human exposure to 121.83  $\mu\text{g}/\text{m}^3$  of air for 24 h. As explained in our previously published  
584 study, such a daily exposure could correspond approximately to that reached during a pollution spike in  
585 the North of France (no data are available for UFPs concentration) (Saleh *et al.*, 2019). This assumption is  
586 possible if no clearance of particles is considered and if the dose of exposure at the lung alveolar  
587 epithelium is not considered (i.e. the same mass deposited on the alveolar epithelial surfaces of mice vs.  
588 humans corresponds to a very different exposure dose in these two species). Moreover, Chen *et al.*  
589 (2010) have shown that in road tunnels, during rush hours (*i.e.* during holidays between 8 am and 11 am),  
590  $\text{PM}_{2.5}$  concentration can increase up to 132  $\mu\text{g}/\text{m}^3$  (46.5  $\mu\text{g}/\text{m}^3$  for  $\text{PM}_{0.1}$ ). According to the very recent  
591 update (April 2018) of the WHO ambient (outdoor) air quality database containing average annual  
592 estimates for  $\text{PM}_{2.5}$  exposure globally (covering more than 4000 cities in 108 countries), the concentration  
593 of atmospheric  $\text{PM}_{2.5}$  of 121.83  $\mu\text{g}/\text{m}^3$  corresponds to a moderate to high exposure to these particles. In all  
594 regions of the world, apart from the high-income region of the Americas, populations are exposed to levels  
595 of  $\text{PM}_{2.5}$  that are higher than the annual mean value of 10  $\mu\text{g}/\text{m}^3$  recommended in the WHO air quality  
596 guideline. In addition, during air pollution peak episodes, as  $\text{PM}_{2.5}$  concentration frequently exceeds 10  
597 times the annual average, we have chosen to expose mice to 100  $\mu\text{g}$  for the single treatment. Therefore,  
598 the doses of 10  $\mu\text{g}$  and 100  $\mu\text{g}$  used in our study for chronic and acute exposures, respectively, are  
599 realistic compared to the different  $\text{PM}_{2.5}$  concentration scenarios found in the atmosphere..

600 Our exhaustive investigation were realized in BALB/c mice after intranasal administration which provides  
601 homogeneous pulmonary delivery of particles and causes pulmonary inflammation (increase in IL-6 level  
602 in plasma) and tissue remodeling at the molecular, cellular and histological level, as observed in our  
603 previously published study (Saleh *et al.*, 2019). Our results revealed that UFPs and FPs induced no  
604 biologically and no statistically significant increases in DNA strand breaks, chromosomal aberrations and  
605 gene mutations, whatever the duration of exposure. Therefore, these airborne particles are considered  
606 having no genotoxic/mutagenic activity under our experimental conditions. These negative results,  
607 although they may seem surprising knowing that PM is classified as carcinogenic, may be explained by  
608 the metabolization and inactivation of mutagenic compounds before they can reach the target cells. In two  
609 old studies, similar negative results were found. Crebelli *et al.* (1988) performed a micronucleus test on  
610 bone marrow in Swiss mice exposed to EOM- $\text{PM}_{10}$  by intraperitoneal (i.p.) injection. No increase in



611 micronuclei frequency was observed. Sprague-Dawley rats were also treated with EOM-PM<sub>10</sub> by gastric  
612 intubation or i.p. injection in order to study body-fluid (blood, urine and feces) mutagenicity (Ames test on  
613 TA98 strain). Negative results were also obtained. This study demonstrated the rapid and total  
614 disappearance of mutagens and pro-mutagens in body fluids of treated animals and thus suggesting an  
615 extensive *in vivo* inactivation, and/or a probably limited bioavailability of mutagens adsorbed onto airborne  
616 PM. Krishna *et al.* (1986) showed no significant increase in the sister-chromatid exchange (SCE) assay  
617 performed in mouse bone marrow and spleen cells after airborne PM oral or i.p. administration. The  
618 authors suggested that it may be due to the inactivation of mutagenic compounds in the body, the inability  
619 of mutagenic compounds to reach the bone marrow and spleen, or the relative low concentration of  
620 mutagenic material in these organs to cause significant increase in SCE level. Furthermore, some studies  
621 reported a cancer promoter effect of air pollution in mice, which could also explain our negative results  
622 obtained in “healthy” cells and organs (Cury *et al.*, 2000; Reymão *et al.*, 1997). Low levels of air pollution  
623 are able to favor the development of tumors, *i.e.* to accelerate the process of progression towards  
624 malignancy. This suggests that mechanisms of mutagenesis may not be directly involved in the induction  
625 of lung cancer, and therefore that FPs and UFPs would probably not be initiating agents at this dose level.

626

627 It is important to highlight the original nature of our work which provides a complete study of the *in vitro*  
628 and *in vivo* genotoxic/mutagenic potential of FPs and UFPs. However, in order to continue to investigate  
629 the mechanism of action of these particles in the appearance and/or development of lung cancer, further  
630 studies would be implemented.

631 For *in vitro* assays, we used three different lung cell models (primary and immortalized cells), cultured  
632 both in submerged conditions and at the ALI. Cells were exposed to FPs and UFPs suspensions which  
633 have advantages (control of the dose actually used, relatively easy to perform, moderate cost...) but  
634 unfortunately also limitations (Fröhlich and Salar-Behzadi, 2014). Indeed, suspension exposure remains a  
635 non-physiological exposure and it is well known that NPs (and PM) suspended in medium often form  
636 aggregates. Therefore, for further refinement of our study it would be interesting to expose cells with  
637 aerosols generated by commercially available ALI-based exposure systems (such as VITROCELL®).  
638 Moreover, as the lung is composed of approximately 40 cell types, a co-culture model with bronchial and  
639 immune (macrophages, monocytes...) cells could be used in order to preserve biological conditions and  
640 intercellular signaling. It is also known that PM can induce oxidative stress via ROS generation through a

641 Fenton-type reaction involving transition metals (and more particularly Fe) but also through reactions  
642 involving organic compounds, such as PAHs, dioxins and furans (André *et al.*, 2011; Baulig *et al.*, 2009;  
643 Boubilil *et al.*, 2013; Kim *et al.*, 2017). We can suppose that the "standard" comet assay, as performed in  
644 our study, may not be sensitive enough to detect oxidative damage. The modified version of the comet  
645 assay using endonucleases (*e.g.* Fpg, hOgg1 or Endo III) could have been useful, as shown by Oh *et al.*  
646 (2011). At last, since air pollution appears to be responsible for increasing the incidence and aggravation  
647 of pulmonary diseases, our present data could be supplemented by toxicological study of FPs and UFPs  
648 on sensitive experimental models such as human bronchial epithelial cells from patients with COPD or  
649 asthma.

650 Regarding our *in vivo* study, we performed a full assessment of the genotoxicity of FPs and UFPs by  
651 performing 3 different tests (comet assay, micronucleus test and Pig-A test), while only one is normally  
652 recommended by the French national agency of medicine and health products safety for drugs in  
653 nanoparticulate form (AFSSAPS, 2011). Nevertheless, our study could be improved by the use of another  
654 mode of treatment. Indeed, mice were treated by intranasal instillation. Although not invasive and not  
655 requiring deep anesthesia, this technique does not correspond to a physiological route of exposure. It  
656 would be interesting to carry out further investigations with systems such as whole-body or nose only  
657 exposure (Fröhlich and Salar-Behzadi, 2014).

658

659 **5. CONCLUSION**

660 To conclude, this work aimed to provide new relevant insights about the possible role of the genotoxicity  
661 and mutagenicity of airborne FPs and UFPs, from an industrial-influenced urban area, in lung  
662 carcinogenesis. We have developed a relevant experimental strategy allowing us to be closer to human  
663 mode of exposure (*i.e.* native PM, ALI exposed cells, animals treated by pulmonary route) and levels of  
664 exposure (*i.e.* moderate doses). Under these experimental conditions, FPs and UFPs are devoid of *in vitro*  
665 genotoxic effect in NHBE primary cells, which therefore seem to be more predictive of an *in vivo* effect. In  
666 return, positive results were obtained in the comet assay in BEAS-2B and NCI-H292 immortalized cell  
667 lines. Our work suggests the need to develop appropriate and standard methodologies for assessing the  
668 *in vitro* genotoxicity of PM. Moreover, FPs and UFPs exhibit no *in vivo* genotoxic/mutagenic activity in our  
669 tested conditions. No differences appear between both types of particles. However, UFPs are known to be  
670 relatively more toxic than larger FPs because they can reach deeper in the airways thanks to their lower  
671 aerodynamic diameter. These data suggest the potential promotion role of FPs and UFPs in pulmonary  
672 carcinogenesis at this dose level, and thus the involvement of other mechanisms than genotoxicity, such  
673 as epigenetic alterations.

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677 **6. CONFLICT OF INTEREST**

678 The authors declare no conflict of interest.

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691 **8. REFERENCES**

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993 **TABLE AND FIGURE LEGENDS**

994

995 **Figure 1. Results of *in vitro* comet assays.**

996 Primary DNA damage was determined in BEAS-2B, NCI-H292 and NHBE cells with the alkaline comet  
997 assay immediately after 4 h exposure to 5 µg PM/cm<sup>2</sup> of airborne particles, at the ALI. Values are depicted  
998 as means of medians of % of tail intensity and standard deviations. Cytotoxicity was assessed at harvest  
999 using the Trypan Blue dye exclusion assay. Results are expressed as relative percent of cell survival (*i.e.*  
1000 the percent ratio of viable unstained cells to non-viable stained cells in exposed versus control groups).  
1001 The control groups were treated with culture medium. MMS was used as positive control (0.96 µg/cm<sup>2</sup>). \*  
1002 p<0.05 (Mann-Whitney U-test). One experiment, 2 biological replicates, 4 technical replicates.

1003

1004 **Figure 2. Results of *in vitro* micronucleus tests.**

1005 Chromosomal aberrations were determined with the micronucleus test after 24 h exposure to 5 µg PM/cm<sup>2</sup>  
1006 of airborne particles in submerged conditions, followed by a 2 days (BEAS-2B and NCI-H292 cells) or a 5  
1007 days (NHBE cells) recovery period. Values are depicted as means of number of micronuclei (MN) per  
1008 1000 cells and standard deviations. Cytotoxicity was evaluated by calculating the percentage of relative  
1009 population doubling (RPD). The control groups were treated with culture medium. MMC was used as  
1010 positive control (0.01 µg/cm<sup>2</sup>). \* p<0.05 (CHI-squared test). One experiment, 2 biological replicates, 4  
1011 technical replicates.

1012

1013 **Figure 3. Results of *in vivo* genotoxicity assessment after acute exposure of airborne particles in**  
1014 **mice.**

1015 Animals (n=5) received 2 treatments at 24-hour interval at the dose of 100 µg of particles. The control  
1016 group received 37 µL of HBSS. MMS was used as positive control [(100 mg/kg b.w./day)x2]. The level of  
1017 primary DNA damage is expressed as the mean of medians of % of tail DNA intensity. \*\* p<0.01 (Mann-  
1018 Whitney U-test).

1019

1020 **Figure 4. Results of *in vivo* genotoxicity/mutagenicity assessment after subacute exposure of**  
1021 **airborne particles in mice.**

1022 Animals (n=5) received 10 µg of particles, 3 times a week, during 1 month. The control group received 37  
1023 µL of HBSS at each treatment. MMS [(100 mg/kg b.w./day)x2] and ENU [(40 mg/kg b.w./day)x3] were  
1024 used as positive controls.

1025 (A - B) The level of primary DNA damage is expressed as the mean of medians of % of tail DNA intensity.

1026 (C) The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic  
1027 erythrocytes (MNPCE) per 1000 cells. (D) The gene mutations frequency is expressed as the number of  
1028 red blood cells (RBC) or reticulocytes (RET) per 10<sup>6</sup> cells. The polychromatic erythrocytes (PCE) /  
1029 normochromatic erythrocytes (NCE) ratio and % RET are used as a measure of bone marrow cytotoxicity.

1030 \* p<0.5; \*\* p<0.01 (Mann-Whitney U-test for the comet assay and the micronucleus test, Dunnet's t-test for  
1031 the Pig-A test).

1032

1033 **Figure 5. Results of *in vivo* genotoxicity/mutagenicity assessment after subchronic exposure of**  
1034 **airborne particles in mice.**

1035 Animals (n=5) received 10 µg of particles, 3 times a week, during 3 months. The control group received 37  
1036 µL of HBSS at each treatment. CPA [(50 mg/kg b.w./day)x1] and ENU [(40 mg/kg b.w./day)x3] were used  
1037 as positive controls.

1038 (A) The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic  
1039 erythrocytes (MNPCE) per 1000 cells. (B) The gene mutations frequency is expressed as the number of  
1040 red blood cells (RBC) or reticulocytes (RET) per 10<sup>6</sup> cells. The polychromatic erythrocytes (PCE) /  
1041 normochromatic erythrocytes (NCE) ratio and % RET are used as a measure of bone marrow cytotoxicity.

1042 \* p<0.5; \*\* p<0.01 (Mann-Whitney U-test for the micronucleus test, Dunnet's t-test for the Pig-A test).

1043

1044 **Table 1. Summary of *in vitro* genotoxic/mutagenic tests performed.**

1045 Cells were treated with 5 µg PM/cm<sup>2</sup> of airborne FPs and UFPs. Comet assay was performed immediately  
1046 after 4 hours exposure at the ALI whereas micronucleus test was performed after 24 hours exposure in  
1047 submerged conditions followed by a 2 days (BEAS-2B and NCI-H292 cells) or a 5 days (NHBE cells)  
1048 recovery period. X: test performed.

1049

1050 **Table 2. Characteristics of NHBE cells used in this study.**

1051 NHBE cells (Lonza) were isolated from the epithelial lining of the airway above bifurcation of the lungs of  
1052 normal donors who have not been diagnosed asthma, COPD or cystic fibrosis.

1053

1054 **Table 3. Summary of in vivo genotoxic/mutagenic tests performed.**

1055 For acute exposure (24 hours), mice received 2 treatments at 24-hours intervals at the dose of 100 µg of  
1056 particles. For subacute (1 month) and subchronic (3 months) exposures, animals were treated with 10 µg  
1057 of particles, 3 times a week. X: test performed.

1058

1059 **Table 4. Summary of in vitro genotoxicity/mutagenicity tests results.**

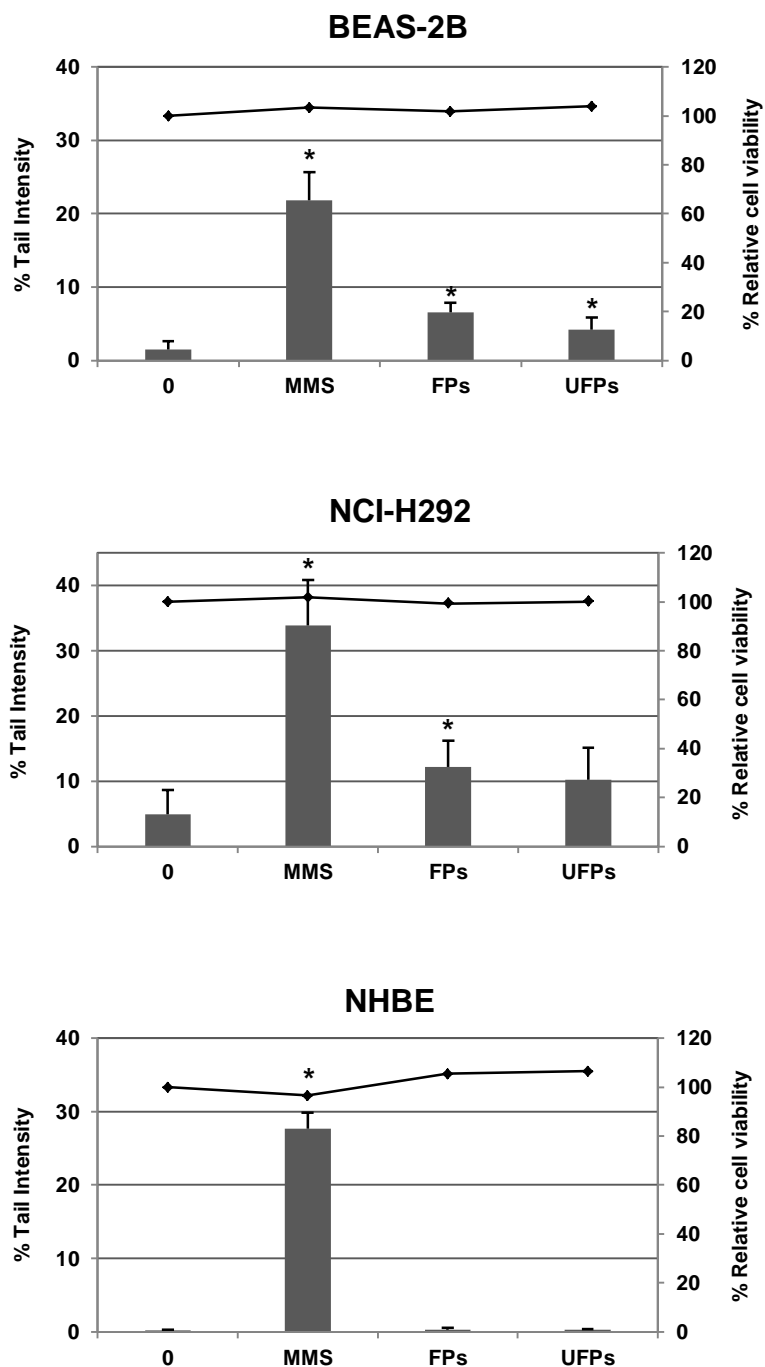
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1063 -: negative result; +: positive result.

1064

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1066 For acute exposure, mice received 2 treatments at 24-hours interval at the dose of 100 µg of particles. For  
1067 subacute (1 month) and subchronic (3 months) exposures, animals were treated with 10 µg of particles, 3  
1068 times a week. n.a.: not assessed; -: negative result.

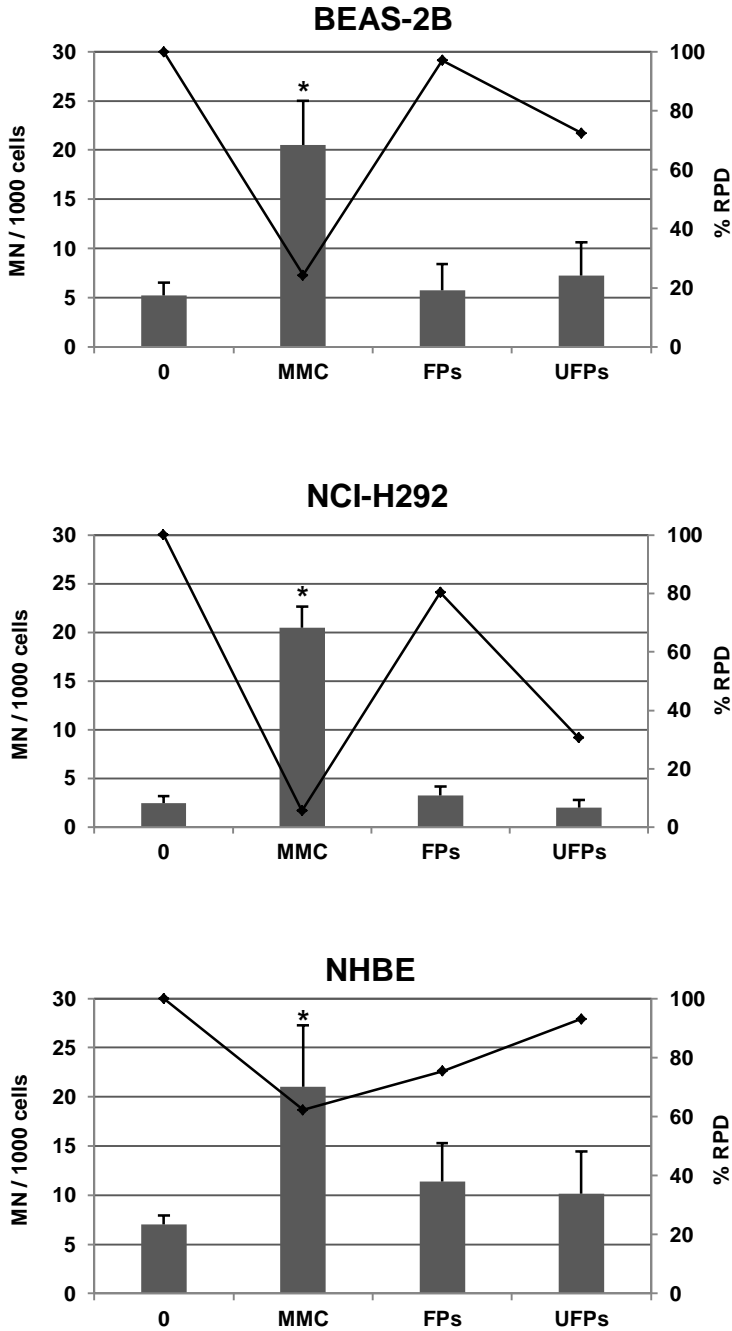
FIGURE 1



**Figure 1. Results of *in vitro* comet assays.**

Primary DNA damage was determined in BEAS-2B, NCI-H292 and NHBE cells with the alkaline comet assay immediately after 4 h exposure to 5  $\mu\text{g PM}/\text{cm}^2$  of airborne particles, at the ALI. Values are depicted as means of medians of % of tail intensity and standard deviations. Cytotoxicity was assessed at harvest using the Trypan Blue dye exclusion assay. Results are expressed as relative percent of cell survival (*i.e.* the percent ratio of viable unstained cells to non-viable stained cells in exposed versus control groups). The control groups were treated with culture medium. MMS was used as positive control (0.96  $\mu\text{g}/\text{cm}^2$ ). \*  $p < 0.05$  (Mann-Whitney U-test). One experiment, 2 biological replicates, 4 technical replicates.

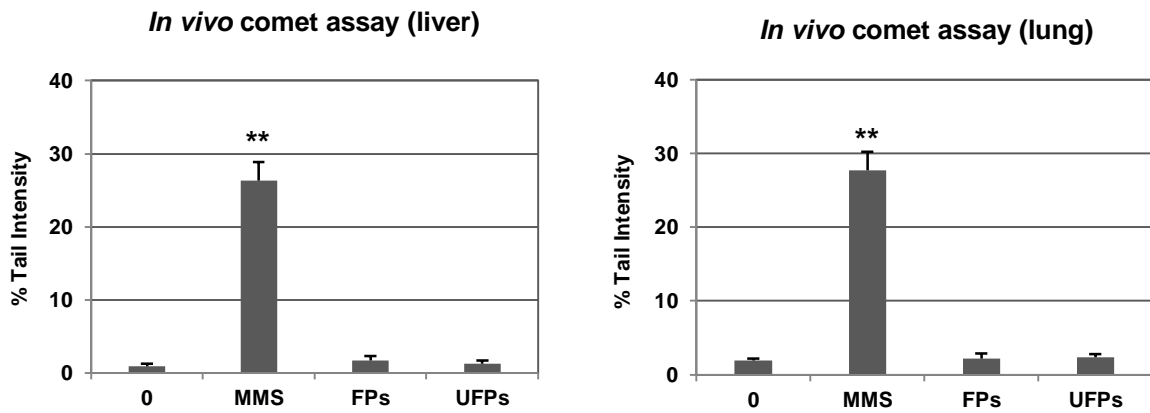
**FIGURE 2**



**Figure 2. Results of *in vitro* micronucleus tests.**

Chromosomal aberrations were determined with the micronucleus test after 24 h exposure to 5 µg PM/cm<sup>2</sup> of airborne particles in submerged conditions, followed by a 2 days (BEAS-2B and NCI-H292 cells) or a 5 days (NHBE cells) recovery period. Values are depicted as means of number of micronuclei (MN) per 1000 cells and standard deviations. Cytotoxicity was evaluated by calculating the percentage of relative population doubling (RPD). The control groups were treated with culture medium. MMC was used as positive control (0.01 µg/cm<sup>2</sup>). \* p<0.05 (CHI-squared test). One experiment, 2 biological replicates, 4 technical replicates.

**FIGURE 3**

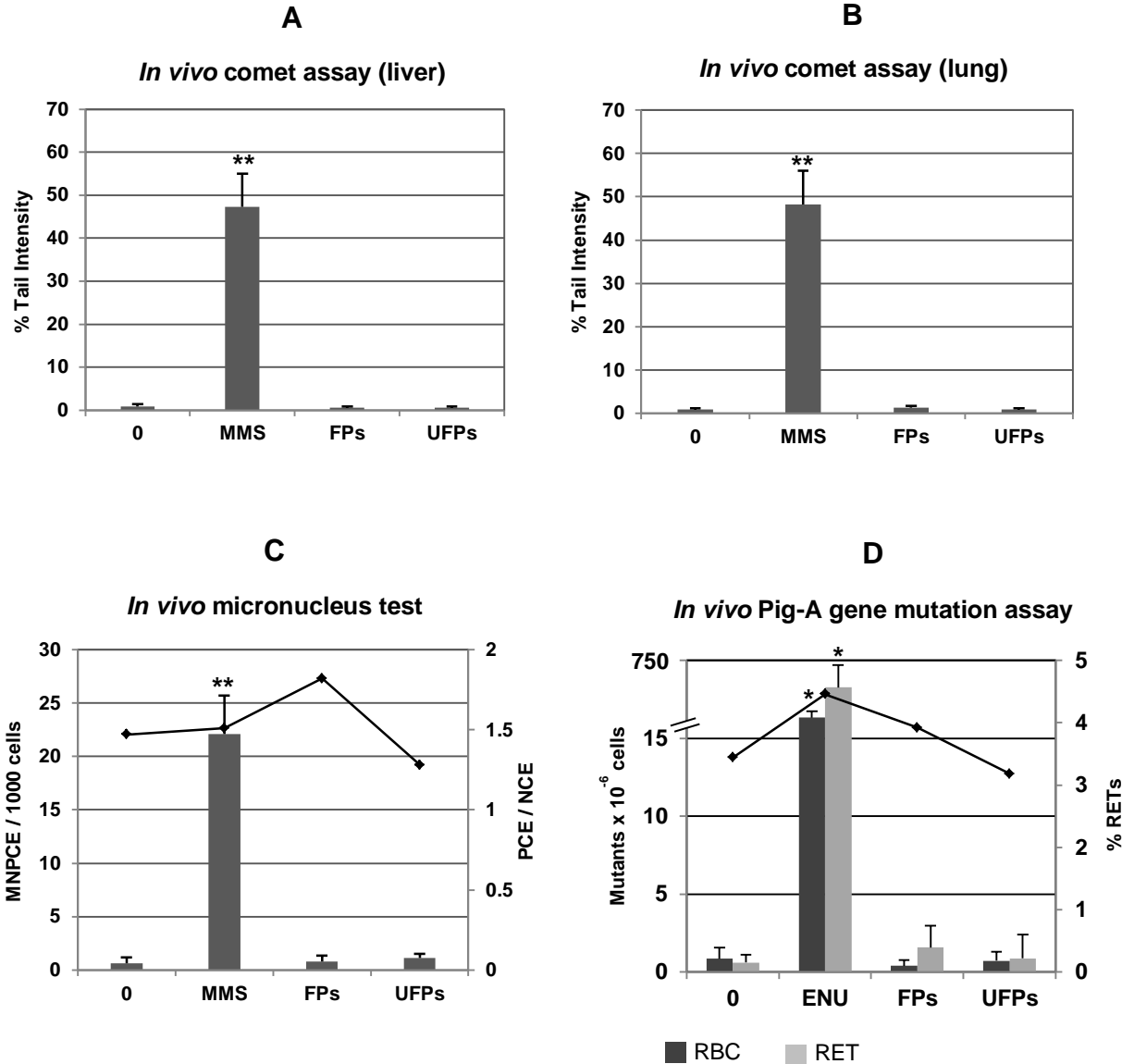


**Figure 3. Results of *in vivo* genotoxicity assessment after acute exposure of airborne particles in mice.**

Animals (n=5) received 2 treatments at 24-hour interval at the dose of 100  $\mu$ g of particles. The control group received 37  $\mu$ L of HBSS. MMS was used as positive control [(100 mg/kg b.w./day)x2]. The level of primary DNA damage is expressed as the mean of medians of % of tail DNA intensity. \*\* p<0.01 (Mann-Whitney U-test).



**FIGURE 4**

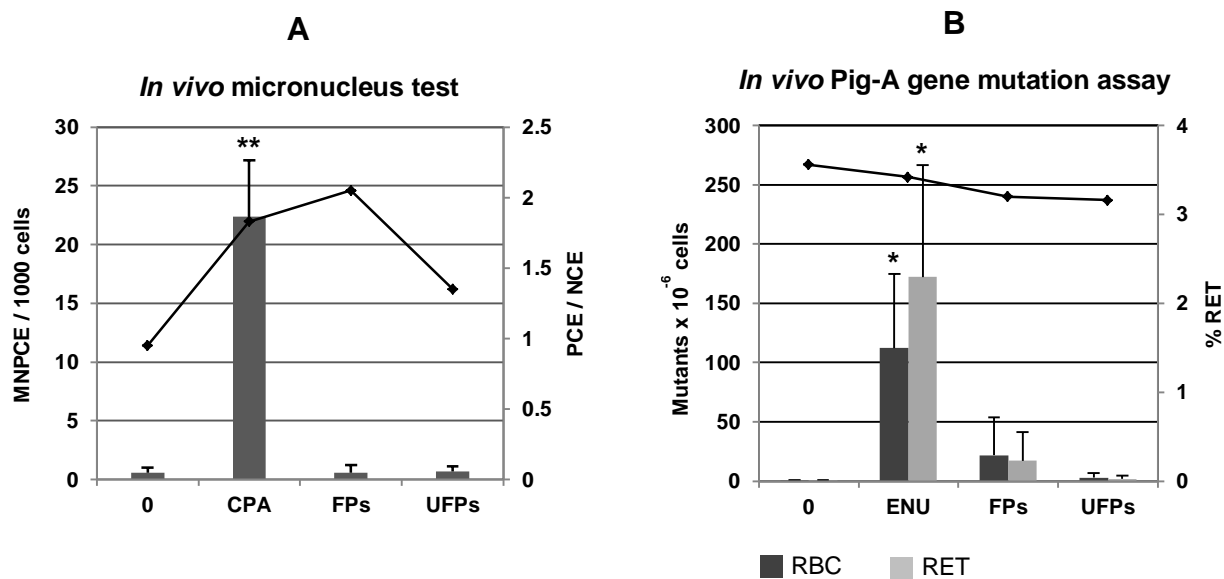


**Figure 4. Results of *in vivo* genotoxicity/mutagenicity assessment after subacute exposure of airborne particles in mice.**

Animals (n=5) received 10 µg of particles, 3 times a week, during 1 month. The control group received 37 µL of HBSS at each treatment. MMS [(100 mg/kg b.w./day)x2] and ENU [(40 mg/kg b.w./day)x3] were used as positive controls.

(A - B) The level of primary DNA damage is expressed as the mean of medians of % of tail DNA intensity. (C) The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic erythrocytes (MNPCE) per 1000 cells. (D) The gene mutations frequency is expressed as the number of red blood cells (RBC) or reticulocytes (RET) per 10<sup>6</sup> cells. The polychromatic erythrocytes (PCE) / normochromatic erythrocytes (NCE) ratio and % RET are used as a measure of bone marrow cytotoxicity. \* p<0.5; \*\* p<0.01 (Mann-Whitney U-test for the comet assay and the micronucleus test, Dunnet's t-test for the Pig-A test).

FIGURE 5



**Figure 5. Results of *in vivo* genotoxicity/mutagenicity assessment after subchronic exposure of airborne particles in mice.**

Animals (n=5) received 10 µg of particles, 3 times a week, during 3 months. The control group received 37 µL of HBSS at each treatment. CPA [(50 mg/kg b.w./day)x1] and ENU [(40 mg/kg b.w./day)x3] were used as positive controls.

(A) The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic erythrocytes (MNPCE) per 1000 cells. (B) The gene mutations frequency is expressed as the number of red blood cells (RBC) or reticulocytes (RET) per 10<sup>6</sup> cells. The polychromatic erythrocytes (PCE) / normochromatic erythrocytes (NCE) ratio and % RET are used as a measure of bone marrow cytotoxicity. \* p<0.5; \*\* p<0.01 (Mann-Whitney U-test for the micronucleus test, Dunnet's t-test for the Pig-A test).

**TABLE 1****Table 1 : Summary of *in vitro* genotoxic/mutagenic tests performed.**

<i>In vitro</i> tests	End-points	Treatment conditions		Cell types		
		Culture	Duration	NHBE	BEAS-2B	NCI-H292
<b>Comet assay</b>	Primary DNA damage	ALI	4 hours	X	X	X
<b>Micronucleus test</b>	Chromosomal aberrations	Immersion	24 hours	X	X	X

Cells were treated with 5 µg PM/cm<sup>2</sup> of airborne FPs and UFPs. Comet assay was performed immediately after 4 hours exposure at the ALI whereas micronucleus test was performed after 24 hours exposure in submerged conditions followed by a 2 days (BEAS-2B and NCI-H292 cells) or a 5 days (NHBE cells) recovery period. X: test performed.

**TABLE 2****Table 2: Characteristics of NHBE cells used in this study.**

<b>SPECIFICATIONS</b>	<b>RESULTS</b>
<b>Donor characteristics</b>	
Age (years)	49
Sex	Female
Race	Caucasian
Smoking habits	Non-smoker
Alcohol use	Non-alcohol user
<b>Virus Testing</b>	
HIV Test	Not detected
HBV Test	Not detected
HCV Test	Not detected
<b>Microbial Testing</b>	
Sterility Test	Negative
Mycoplasma	Negative
<b>Cell Performance Testing</b>	
Cell passage frozen	1
Viability	84 %
Cell count (cells/vial)	879 000
Seeding efficiency	27 %
Doubling time (hours)	28

NHBE cells (Lonza) were isolated from the epithelial lining of the airway above bifurcation of the lungs of normal donors who have not been diagnosed asthma, COPD or cystic fibrosis.

**TABLE 3****Table 3 : Summary of *in vivo* genotoxic/mutagenic tests performed.**

<i>In vivo</i> tests	End-points	Target organs	Treatment schedule		
			24 hours	1 month	3 months
<b>Comet assay</b>	Primary DNA damage	Liver, lung	X	X	
<b>Micronucleus test</b>	Chromosomal aberrations	Bone marrow		X	X
<b>Pig-A gene mutation assay</b>	Gene mutations	Erythrocytes		X	X

For acute exposure (24 hours), mice received 2 treatments at 24-hours intervals at the dose of 100 µg of particles. For subacute (1 month) and subchronic (3 months) exposures, animals were treated with 10 µg of particles, 3 times a week. X: test performed.

**TABLE 4****Table 4 : Summary of *in vitro* genotoxicity/mutagenicity tests results.**

<i>In vitro</i> tests	End-points	Treatment conditions	Cell types		
			NHBE	BEAS-2B	NCI-H292
<b>Comet assay</b>	Primary DNA damage	ALI	-	+	+
<b>Micronucleus test</b>	Chromosomal aberrations	Immersion	-	-	-

Cells were treated with 5 µg/cm<sup>2</sup> of airborne FPs and UFPs. Comet assay was performed immediately after 4h exposure at the ALI whereas micronucleus test was performed after 24h exposure in submerged conditions followed by a 2 days (BEAS-2B and NCI-H292 cells) or a 5 days (NHBE cells) recovery period. -: negative result; +: positive result.

**TABLE 5****Table 5 : Summary of *in vivo* genotoxicity/mutagenicity tests results.**

<i>In vivo</i> tests	End-points	Target organs	Treatment schedule		
			24 hours	1 month	3 months
<b>Comet assay</b>	Primary DNA damage	Liver, lung	-	-	n.a.
<b>Micronucleus test</b>	Chromosomal aberrations	Bone marrow	n.a.	-	-
<b>Pig-A gene mutation assay</b>	Gene mutations	Erythrocytes	n.a.	-	-

For acute exposure, mice received 2 treatments at 24-hours interval at the dose of 100 µg of particles. For subacute (1 month) and subchronic (3 months) exposures, animals were treated with 10 µg of particles, 3 times a week. n.a.: not assessed; -: negative result.