



Study of in vitro and in vivo genotoxic effects of air pollution fine (PM_{2.5-0.18}) and quasi-ultrafine (PM_{0.18}) particles on lung models

A. Platel, K. Privat, S. Talahari, A. Delobel, G. Dourdin, E. Gateau, S. Simar, Y. Saleh, J. Sotty, S. Antherieu, et al.

► To cite this version:

A. Platel, K. Privat, S. Talahari, A. Delobel, G. Dourdin, et al.. Study of in vitro and in vivo genotoxic effects of air pollution fine (PM_{2.5-0.18}) and quasi-ultrafine (PM_{0.18}) particles on lung models. Science of the Total Environment, 2020, 711, pp.134666. 10.1016/j.scitotenv.2019.134666 . hal-02913612

HAL Id: hal-02913612

<https://hal.science/hal-02913612>

Submitted on 7 Mar 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Study of *in vitro* and *in vivo* genotoxic effects of air pollution fine (PM_{2.5-0.18}) and**
2 **quasi-ultrafine (PM_{0.18}) particles on lung models**

3

4	A. Platel ^{a,*}	anne.platel@pasteur-lille.fr
5	K. Privat ^a	killian.privat@yahoo.fr
6	S.Talahari ^a	smail.talahari@pasteur-lille.fr
7	A. Delobel ^a	alexandre.delobel@pasteur-lille.fr
8	G. Dourdin ^a	gonzague.dourdin@pasteur-lille.fr
9	E. Gateau ^a	eulalie.gateau@pasteur-lille.fr
10	S. Simar ^a	sophie.simar@pasteur-lille.fr
11	Y. Saleh ^a	yara.saleh@univ-lille.fr
12	J. Sotty ^a	jules.sotty@univ-lille.fr
13	S. Antherieu ^a	sebastien.antherieu@univ-lille.fr
14	L. Canivet ^a	ludivine.canivet@univ-lille.fr
15	L.-Y. Alleman ^b	laurent.alleman@imt-lille-douai.fr
16	E. Perdrix ^b	esperanza.perdrix@imt-lille-douai.fr
17	G. Garçon ^a	guillaume.garcon@univ-lille.fr
18	F.O. Denayer ^a	franck-olivier.denayer@univ-lille.fr
19	J.M. Lo Guidice ^a	jean-marc.lo-guidice@univ-lille.fr
20	F. Nesslany ^a	fabrice.nesslany@pasteur-lille.fr

21

22 ^a Université de Lille, CHU Lille, Institut Pasteur de Lille, EA4483-IMPECS, France
23 (Laboratoire de Toxicologie Génétique, Institut Pasteur de Lille, 1 rue du Professeur Calmette, 59000 Lille,
24 France)

25

26 ^b IMT Lille Douai, Univ. Lille, SAGE - Département Sciences de l'Atmosphère et Génie de
27 l'Environnement, 59000 Lille, France
28 (Ecole des Mines de Douai, Département Chimie et Environnement, 941 rue Charles Bourseul, BP 10838,
29 59508 Douai cedex, France)

30

31 * Corresponding author:
32 Anne PLATEL
33 Laboratoire de Toxicologie Génétique
34 Institut Pasteur de Lille
35 1 rue du Professeur Calmette
36 59000 Lille
37 FRANCE
38 anne.platel@pasteur-lille.fr
39

ABSTRACT

Air pollution and particulate matter (PM) are classified as carcinogenic to humans . Pollutants evidence for public health concern include coarse (PM₁₀) and fine (PM_{2.5}) particles. However, ultrafine particles (PM_{0.1}) are assumed to be more toxic than larger particles, but data are still needed to better understand their mechanism of action.

In this context, the aim of our work was to investigate the *in vitro* and *in vivo* genotoxic potential of fine (PM_{2.5-0.18}) and quasi ultra-fine (PM_{0.18}) particles from an urban-industrial area (Dunkirk, France) by using comet, micronucleus and/or gene mutation assays. *In vitro* assessment was performed with 2 lung immortalized cell lines (BEAS-2B and NCI-H292) and primary normal human bronchial epithelial cells (NHBE) grown at the air-liquid interface or in submerged conditions (5 µg PM/cm²). For *in vivo* assessment, tests were performed after acute (24 hours, 100 µg PM/animal), subacute (1 month, 10 µg PM/animal) and subchronic (3 months, 10 µg PM/animal) intranasal exposure of BALB/c mice.

In vitro, our results show that PM_{2.5-0.18} and PM_{0.18} induced primary DNA damage but no chromosomal aberrations in immortalized cells. Negative results were noted in primary cells for both endpoints. *In vivo* assays revealed that PM_{2.5-0.18} and PM_{0.18} induced no significant increases in DNA primary damage, chromosomal aberrations or gene mutations, whatever the duration of exposure.

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

KEYWORDS

Fine and ultrafine particles

Air pollution

Human lung cell models

Animal model

Genotoxicity

1. INTRODUCTION

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

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

In this context, following our previous work on the *in vitro* genotoxicity of PM_{2.5} from an urban surrounding (Leclercq *et al.*, 2017), the aim of our work was to investigate the *in vitro* and *in vivo* genotoxic and mutagenic potential of air pollution FPs (2.5 µm - 0.18 µm) and quasi UFPs (≤ 0.18 µm) collected in the industrial-port area of Dunkirk (Northern France), near the motorways and the conurbation. For a better human health assessment, we strove to be as close as possible to the actual *in vivo* exposure conditions in terms of route of exposure. For that, the collected particles were directly tested on cells or administered to animals, rather than testing the extracted organic matter (EOM) of the PM, as frequently reported in the literature for genotoxicity assessment (Abbas *et al.*, 2019; Bełcik *et al.*, 2018; Billet *et al.*, 2018; Bocchi *et al.*, 2017, 2016; Borgie *et al.*, 2015; Cassoni *et al.*, 2004; Gábelová *et al.*, 2007a, 2007b, 2004; Lemos *et al.*, 2016; Oh *et al.*, 2011; Topinka *et al.*, 2000). *In vitro* assessment was performed with two human lung cell models, common immortalized cell lines (BEAS-2B and NCI-H292 cells) and primary cells [normal human bronchial epithelial cells (NHBE)], by using the *in vitro* comet and micronucleus assays at the dose of 5 µg PM/cm². These tests have been chosen for their complementarities for the detection of primary DNA damage and chromosomal aberrations, respectively. These two methodologies appear to be well adapted to highlight a broad spectrum of genetic and mutagenic events, particularly of clastogenic and aneugenic types. It is known that after nanoparticles exposure, the comet assay is the most common test for DNA damage detection, and that the micronucleus test is less time-consuming and thus more used for nanoparticles testing than the chromosomal aberration assay (Dusinska *et al.*, 2017). Moreover, they represent the most commonly used combination of tests for nanoparticles genotoxicity studies (Magdolenova *et al.*, 2014). From a methodological point of view, the comet assay was performed on cells exposed at the air-liquid interface (ALI) in order to obtain a differentiation state as close as possible to the cellular exposure conditions observed *in vivo*. ALI cell culture is a useful method for investigating the harmful effects of PM on the respiratory airway as primary cells exhibit differentiation and pseudostratified striation similar to those observed *in vivo* (Cho *et al.*, 2018). On the other hand, the micronucleus test

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

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

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

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

2.2. Particles sampling

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

2.3. Physicochemical characterization

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

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

2.4. *In vitro* genotoxicity and cytotoxicity assessment

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

2.4.1. Preparation of particles suspensions

Firstly, FP and UFP samples were suspended in HBSS. Then, these initial particles suspensions were prepared following a protocol described by Jensen *et al.* in the NANOGENOTOX Joint Action (Jensen *et al.*, 2011). Briefly, UFP and FPs were suspended in 0.05% BSA-water in order to obtain a 10x initial particles solution (BSA-water was prepared by dissolving 0.05% w/v BSA in distilled water and sterile-filtered) and dispersed by probe-sonication for 16 min, at 400W and 10% amplitude using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA). UFPs and FPs suspensions were cooled on ice to minimize heat-development during sonication. These initial suspensions were then diluted 1:9 v/v in cell culture medium, supplemented with amphotericin B (20 mg/mL) at 2% (v/v), at the time of cell treatment to obtain final suspensions at 5 µg PM/cm². Initial and final suspensions were carefully vortexed before each pipetting.

2.4.2. Cell lines and culture conditions

2.4.2.1. Primary cells: NHBE cells

NHBE cells (Lonza, Verviers, Belgium) were isolated from donated human tissue (from epithelial lining of airways above bifurcation of the lungs), after obtaining permission for their use in research applications by informed consent or legal authorization. The characteristics of the healthy donor and cells are shown in Table 2. Cells were cultured in 75 cm² Corning® CellBIND® surface cell culture flasks (Sigma-Aldrich, St. Quentin Fallavier, France) in bronchial epithelial growth medium BEGM according to the manufacturer recommendations. Medium was renewed every 3 days and cells were subcultured once a week and seeded at a density of 5x10⁵ cells per flask. The doubling time of NHBE cells was approximately 30 hours

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

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

NCI-H292 cells. The human muco-epidermoid bronchiolar carcinoma cell line NCI-H292 was obtained from the American Type Culture Collection (ATCC® CRL-1848™). Cells were seeded at a density of 5×10^5 cells per culture flask (75 cm²; Falcon™, Becton Dickinson Biosciences, France) and maintained at 37°C in a humidified 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 200 UI/mL penicillin, 50 mg/L streptomycin, 2.5 mg/L amphotericin B. This medium is hereafter referred to as RPMI 10 growth medium. Medium was renewed every 2 to 3 days and cells were subcultured when they reached 80-90 % confluence. The ATCC indicates that the doubling time of NCI-H292 cells is approximately 48 hours.

BEAS-2B cells. The human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (ATCC® CRL-9609™). Comparable to the NHBE cells, BEAS-2B cells were seeded at a density of 5×10^5 cells in 75 cm² Corning® CellBIND® surface cell culture flasks and maintained at 37°C in a humidified 5% CO₂ atmosphere in BEGM. Medium was renewed every 2 to 3 days and cells were subcultured before reaching confluence since confluent cultures rapidly undergo squamous terminal differentiation. The doubling time of BEAS-2B cells is approximately 24 hours (Costa *et al.*, 2010; Haniu *et al.*, 2013).

2.4.3. In vitro comet assay

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

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

replaced by B-ALI™ differentiation medium with 0.2% (v/v) B-ALI™ inducer. For NHBE cells, differentiation at the ALI is reached after 21 days of culture. This effective differentiation was already checked by Leclercq *et al.* (2016) and Sotty *et al.* (2019). One experiment was performed with duplicate cultures (*i.e.* 2 biological replicates) for each group (*i.e.* 2 wells per dose). Each treatment (without metabolic activation) was coupled to an assessment of cytotoxicity as described below. In each insert, the apical medium was removed to expose the cells. Particles suspensions (as described in § 2.4.1) were then directly deposited by pipetting on the permeable membrane inserts (300 µL/insert), for 4 h without recovery time (4h+0h). Then cells were washed twice with PBS and immediately harvested by trypsinization with 1 mL/well of trypsin-EDTA for NHBE cells or 1 mL/well of TrypLE™ Express for BEAS-2B and NCI-H292 cells, for 4 min at 37°C. Then, 1 mL/well of Trypsin Neutralizing Solution for NHBE cells, 1 mL/well of RPMI 10 growth medium for NCI-H292 cells, or 1 mL/well of BEGM for BEAS-2B cells, was added in order to stop the action of trypsin. Cells were transferred to 15 mL polypropylene tubes and centrifuged at 1000 rpm for 5 min. Supernatant was discarded and the pelleted cells were immediately used for the comet assay. Positive control was MMS (0.96 µg/cm²) and negative control was culture medium.

2.4.3.2. Comet assay protocol

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

2.4.3.3. Cytotoxicity assessment

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

2.4.4. *In vitro* micronucleus test

2.4.4.1. *Cell treatment in submerged conditions*

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

2.4.4.2. *Micronucleus test protocol*

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

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

2.4.4.3. Cytotoxicity assessment

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

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

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

2.5. *In vivo* genotoxicity assessment

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

2.5.1. Animal model

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

2.5.2. Treatments

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

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

2.5.3. *In vivo* comet assay

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

2.5.4. *In vivo* micronucleus test

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

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

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

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

2.6. Statistical Analysis

For each test, differences with a $p < 0.05$ were considered statistically significant. For the *in vitro* and *in vivo* comet assay, it has previously been shown that the tail intensity does not follow a Gaussian distribution (Bauer *et al.*, 1998). Thus, the non-parametric Mann-Whitney U-test was used to evaluate the statistical difference between groups (*i.e.* between each concentration vs. the respective negative control). All statistical analyses were performed with StatView® Software (version 5.0, SAS 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® 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.

3. RESULTS

3.1. *In vitro* genotoxicity and cytotoxicity assessment

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

3.1.1. *In vitro* comet assay

Primary DNA damage was measured with the *in vitro* comet assay after a 4 h treatment. Results of the means of medians of percentage of tail intensity (TI) for each group and the corresponding relative cell viability obtained in BEAS-2B, NCI-H292 and NHBE cells are presented in Figure 1. To exclude cytotoxicity as a confounding factor, the cell viability should be more than 70 % for the comet assay (Collins *et al.*, 2008; Tice *et al.*, 2000; Witte *et al.*, 2007). Figure 1 shows that both types of airborne particles did not induce any significant cytotoxic effects at the tested concentration of 5 µg PM/cm², as assessed by the trypan blue dye exclusion assay. Moreover, under tested conditions, no increase in DNA strand breaks was observed in NHBE cells, whatever the type of particles. On the contrary, slight but statistically significant increases ($p < 0.05$) in TI were obtained in BEAS-2B cells (6.55 % and 4.19 % for FPS and UFPs, respectively, vs. 1.54 % for the negative control). For NCI-H292 cells, increases in TI were observed for both particles, statistically significant only for FPs [12.24% for FPs ($p < 0.05$) and 10.25% for UFPs, vs. 4.97 % for the negative control].

3.1.2. *In vitro* micronucleus test

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

3.2. *In vivo* genotoxicity assessment

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

3.2.1. Acute exposure

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

3.2.2. Subacute exposure

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

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

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

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

3.2.3. Subchronic exposure

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

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

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

4. DISCUSSION

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

For the *in vitro* approach, we used two types of lung cell models: standard cell lines (BEAS-2B and NCI-H292 cells) and human primary cells (NHBE cells). In order to be as close as possible to the “real” *in vivo* exposure conditions, the comet assay was performed on cells exposed at the ALI. Because of the absolute need of proliferative cells for the study of chromosomal aberrations, the *in vitro* micronucleus test cells were performed on cells grown in submerged conditions (under these conditions, cells could not be differentiated). The choice of the PM concentration for cell exposure was based on preliminary cell viability assays (measures of the amount of intracellular ATP and release of G6PD in the culture medium). The concentration of 5 µg PM/cm² corresponds to the 10% lethal concentration (LC₁₀) (Sotty *et al.*, 2019). This was confirmed by our data, since, in our test conditions, FPs and UFPs did not induce any significant cytotoxic effects, neither in the comet assay nor in the micronucleus test, in the three cell types (except for UFPs which induced *ca.* 70% of cytotoxicity on NCI-H292 in the micronucleus test). We have shown that for primary NHBE cells, negative results were obtained for both endpoints, as observed *in vivo*. As expected, primary cells appeared to be more predictive of an *in vivo* effect. The lack of genotoxic activity observed with NHBE cells was also consistent with our previously published data on the *in vitro*

491 genotoxicity of air pollution-derived PM_{2.5} 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

core of the particles is not assessed. Nevertheless, increases in DNA damage and in chromosomal aberrations were not systematically reported since some negative genotoxicity results were also found in the literature. For example, André *et al.* (2011) have directly tested suspensions of PM_{2.5} collected in Dunkirk. Exposure of A549 immortalized pulmonary cells to PM_{2.5} for 72 h at the LC50 (31.6 µg PM/cm²) did not lead to quantifiable PAH bulky DNA adducts (although A549 cells are cancerous cells with highly active phase I metabolism enzymes). Recently, Billet *et al.* (2018) observed neither the formation of DNA bulky adducts after a 72 h-exposure of BEAS-2B cells to PM_{0.3-2.5} or to PM-EOM, despite the high sensitivity of the ³²P post-labeling used method. Abbas noticed slight but significant decreases of phosphorylated-Histone 2AX (γH2AX) in BEAS-2B cells 72 h after their exposure to the organic fraction of air pollution-derived PM_{2.5} from 3 to 30 µg PM_{2.5}-eq/cm² (Abbas *et al.*, 2019). In return, 8-OHdG adducts were measured as significantly increased after 48 h of treatment but exclusively at the highest concentration of 15 µg PM/cm² (not at 3 µg PM/cm²). However, 8-OHdG formation was shown in A549 cells after incubation for 24 h, 48 h and 72 h with whole PM_{2.5} from Dunkirk at concentrations corresponding to the LC10 (6.33 µg PM/cm²) and LC50 (31.6 µg PM/cm²) (André *et al.*, 2011). Lepers *et al.* (2014) also observed positive responses in the comet and in the micronucleus assays, as well as for histone H2AX phosphorylation, in the BEAS-2B cell line exposed for 72 h to native PM samples from industrial, urban, and rural areas of Dunkirk, and exclusively at the higher concentration tested of 15 µg PM/cm² (no genotoxic effect appeared at the lower dose of 3.75 µg PM/cm²). This suggests that, in our study, the concentration (5 µg PM/cm²) and treatment times (4 h for the comet assay and 24 h for the micronucleus test), although more realistic, were probably not high and not long enough, respectively, to provide measurable genotoxic effects in all cellular models and in all end-points because of their sensitivity. However, we are aware that the PM concentrations we used in our *in vitro* study are quite far from environmental exposure expected in humans. Recently, Gualtieri *et al.* (2018) studied the effects of air pollution on BEAS-2B cells exposed at the ALI under 'real' environmental conditions. They showed that the total theoretically deposited mass, being in the order of 10⁻² ng/cm² is roughly 3 orders of magnitude lower than the doses usually reported in PM *in vitro* toxicological studies performed under laboratory conditions (Gualtieri *et al.*, 2018).

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

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

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

and subchronic exposures, respectively. We have estimated that intratracheal instillation of 10 µg of PM_{2.5} to BALB/c mice [whose minute ventilation is 57 mL/min according to de Hennezel *et al.* (2001)] is equivalent to human exposure to 121.83 µg/m³ of air for 24 h. As explained in our previously published study, such a daily exposure could correspond approximately to that reached during a pollution spike in the North of France (no data are available for UFPs concentration) (Saleh *et al.*, 2019). This assumption is possible if no clearance of particles is considered and if the dose of exposure at the lung alveolar epithelium is not considered (i.e. the same mass deposited on the alveolar epithelial surfaces of mice vs. humans corresponds to a very different exposure dose in these two species). Moreover, Chen *et al.* (2010) have shown that in road tunnels, during rush hours (*i.e.* during holidays between 8 am and 11 am), PM_{2.5} concentration can increase up to 132 µg/m³ (46.5 µg/m³ for PM_{0.1}). According to the very recent update (April 2018) of the WHO ambient (outdoor) air quality database containing average annual estimates for PM_{2.5} exposure globally (covering more than 4000 cities in 108 countries), the concentration of atmospheric PM_{2.5} of 121.83 µg/m³ corresponds to a moderate to high exposure to these particles. In all regions of the world, apart from the high-income region of the Americas, populations are exposed to levels of PM_{2.5} that are higher than the annual mean value of 10 µg/m³ recommended in the WHO air quality guideline. In addition, during air pollution peak episodes, as PM_{2.5} concentration frequently exceeds 10 times the annual average, we have chosen to expose mice to 100 µg for the single treatment. Therefore, the doses of 10 µg and 100 µg used in our study for chronic and acute exposures, respectively, are realistic compared to the different PM_{2.5} concentration scenarios found in the atmosphere..

Our exhaustive investigation were realized in BALB/c mice after intranasal administration which provides homogeneous pulmonary delivery of particles and causes pulmonary inflammation (increase in IL-6 level in plasma) and tissue remodeling at the molecular, cellular and histological level, as observed in our previously published study (Saleh *et al.*, 2019). Our results revealed that UFPs and FPs induced no biologically and no statistically significant increases in DNA strand breaks, chromosomal aberrations and gene mutations, whatever the duration of exposure. Therefore, these airborne particles are considered having no genotoxic/mutagenic activity under our experimental conditions. These negative results, although they may seem surprising knowing that PM is classified as carcinogenic, may be explained by the metabolization and inactivation of mutagenic compounds before they can reach the target cells. In two old studies, similar negative results were found. Crebelli *et al.* (1988) performed a micronucleus test on bone marrow in Swiss mice exposed to EOM-PM₁₀ by intraperitoneal (i.p.) injection. No increase in

micronuclei frequency was observed. Sprague-Dawley rats were also treated with EOM-PM₁₀ by gastric intubation or i.p. injection in order to study body-fluid (blood, urine and feces) mutagenicity (Ames test on TA98 strain). Negative results were also obtained. This study demonstrated the rapid and total disappearance of mutagens and pro-mutagens in body fluids of treated animals and thus suggesting an extensive *in vivo* inactivation, and/or a probably limited bioavailability of mutagens adsorbed onto airborne PM. Krishna *et al.* (1986) showed no significant increase in the sister-chromatid exchange (SCE) assay performed in mouse bone marrow and spleen cells after airborne PM oral or i.p. administration. The authors suggested that it may be due to the inactivation of mutagenic compounds in the body, the inability of mutagenic compounds to reach the bone marrow and spleen, or the relative low concentration of mutagenic material in these organs to cause significant increase in SCE level. Furthermore, some studies reported a cancer promoter effect of air pollution in mice, which could also explain our negative results obtained in “healthy” cells and organs (Cury *et al.*, 2000; Reymão *et al.*, 1997). Low levels of air pollution are able to favor the development of tumors, *i.e.* to accelerate the process of progression towards malignancy. This suggests that mechanisms of mutagenesis may not be directly involved in the induction of lung cancer, and therefore that FPs and UFPs would probably not be initiating agents at this dose level.

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

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

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

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

5. CONCLUSION

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

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. ACKNOWLEDGMENTS

This work benefited grants from the Region Hauts-de-France (PRSE, Convention N°13004366), the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) (PNREST ANSES, CANCER ITMO AVIESAN, Convention N°2014/1/183) and the European Funds for Regional Economical Development (CPER CLIMIBIO). We thank E. Vernet for technical assistance. We thank Dr Helene Bauderlique from the BioImaging Center Lille for facility access to the cytometer BD Canto II (BICeL-campus Pasteur, 1 rue du Prof Calmette, 59019 Lille).

691 8. REFERENCES

- 692
693 Abbas, I., Badran, G., Verdin, A., Ledoux, F., Roumie, M., Lo Guidice, J.-M., Courcot, D., Garçon, G.,
694 2019. In vitro evaluation of organic extractable matter from ambient PM_{2.5} using human bronchial
695 epithelial BEAS-2B cells: Cytotoxicity, oxidative stress, pro-inflammatory response, genotoxicity,
696 and cell cycle deregulation. *Environmental Research* 171, 510–522.
697 <https://doi.org/10.1016/j.envres.2019.01.052>
- 698 AFSSAPS, 2011. Recommandations relatives à l'évaluation toxicologique des médicaments sous forme
699 nanoparticulaires.
- 700 Agence Régionale de Santé des Hauts-de-France, 2017. Diagnostic territorialisé des Hauts-de-France,
701 Territoire de proximité.
- 702 Amini, H., Trang Nhung, N.T., Schindler, C., Yunesian, M., Hosseini, V., Shamsipour, M., Hassanvand,
703 M.S., Mohammadi, Y., Farzadfar, F., Vicedo-Cabrera, A.M., Schwartz, J., Henderson, S.B.,
704 Künzli, N., 2019. Short-term associations between daily mortality and ambient particulate matter,
705 nitrogen dioxide, and the air quality index in a Middle Eastern megacity. *Environmental Pollution*
706 254, 113121. <https://doi.org/10.1016/j.envpol.2019.113121>
- 707 André, V., Billet, S., Pottier, D., Le Goff, J., Pottier, I., Garçon, G., Shirali, P., Sichel, F., 2011.
708 Mutagenicity and genotoxicity of PM_{2.5} issued from an urbano-industrialized area of Dunkerque
709 (France). *Journal of Applied Toxicology* 31, 131–138. <https://doi.org/10.1002/jat.1572>
- 710 Bakand, S., 2016. Cell culture techniques essential for toxicity testing of inhaled materials and
711 nanomaterials in vitro. *J Tissue Sci Eng* 07. <https://doi.org/10.4172/2157-7552.1000181>
- 712 Bauer, E., Recknagel, R.-D., Fiedler, U., Wollweber, L., Bock, C., Greulich, K.O., 1998. The distribution of
713 the tail moments in single cell gel electrophoresis (comet assay) obeys a chi-square (χ^2) not a
714 gaussian distribution. *Mutation Research/Fundamental and Molecular Mechanisms of*
715 *Mutagenesis* 398, 101–110. [https://doi.org/10.1016/S0027-5107\(97\)00246-7](https://doi.org/10.1016/S0027-5107(97)00246-7)
- 716 Baulig, A., Singh, S., Marchand, A., Schins, R., Barouki, R., Garlatti, M., Marano, F., Baeza-Squiban, A.,
717 2009. Role of Paris PM_{2.5} components in the pro-inflammatory response induced in airway
718 epithelial cells. *Toxicology* 261, 126–135. <https://doi.org/10.1016/j.tox.2009.05.007>
- 719 Belcik, M.K., Trusz-Zdybek, A., Zaczynska, E., Czarny, A., Piekarska, K., 2018. Genotoxic and cytotoxic
720 properties of PM_{2.5} collected over the year in Wrocław (Poland). *Science of The Total*
721 *Environment* 637–638, 480–497. <https://doi.org/10.1016/j.scitotenv.2018.04.166>
- 722 Billet, S., Landkocz, Y., Martin, P.J., Verdin, A., Ledoux, F., Lepers, C., André, V., Cazier, F., Sichel, F.,
723 Shirali, P., Gosset, P., Courcot, D., 2018. Chemical characterization of fine and ultrafine PM,
724 direct and indirect genotoxicity of PM and their organic extracts on pulmonary cells. *Journal of*
725 *Environmental Sciences* 71, 168–178. <https://doi.org/10.1016/j.jes.2018.04.022>
- 726 Bocchi, C., Bazzini, C., Fontana, F., Pinto, G., Cassoni, F., 2017. Genotoxicity of airborne PM_{2.5}
727 assessed by salmonella and comet assays in five cities of the Emilia-Romagna (Italy)
728 mutagenicity monitoring network. *Environmental and Molecular Mutagenesis* 58, 719–729.
729 <https://doi.org/10.1002/em.22141>
- 730 Bocchi, C., Bazzini, C., Fontana, F., Pinto, G., Martino, A., Cassoni, F., 2016. Characterization of urban
731 aerosol: seasonal variation of mutagenicity and genotoxicity of PM_{2.5}, PM₁ and semi-volatile
732 organic compounds. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 809,
733 16–23. <https://doi.org/10.1016/j.mrgentox.2016.07.007>
- 734 Borgie, M., Dagher, Z., Ledoux, F., Verdin, A., Cazier, F., Martin, P., Hachimi, A., Shirali, P., Greige-
735 Gerges, H., Courcot, D., 2015. Comparison between ultrafine and fine particulate matter collected
736 in Lebanon: Chemical characterization, in vitro cytotoxic effects and metabolizing enzymes gene
737 expression in human bronchial epithelial cells. *Environmental Pollution* 205, 250–260.
738 <https://doi.org/10.1016/j.envpol.2015.05.027>
- 739 Boubli, L., Assémat, E., Borot, M.-C., Boland, S., Martinon, L., Sciare, J., Baeza-Squiban, A., 2013.
740 Development of a repeated exposure protocol of human bronchial epithelium in vitro to study the
741 long-term effects of atmospheric particles. *Toxicology in Vitro* 27, 533–542.
742 <https://doi.org/10.1016/j.tiv.2012.11.008>
- 743 Burlinson, B., Tice, R.R., Speit, G., Agurell, E., Brendler-Schwaab, S.Y., Collins, A.R., Escobar, P.,
744 Honma, M., Kumaravel, T.S., Nakajima, M., Sasaki, Y.F., Thybaud, V., Uno, Y., Vasquez, M.,
745 Hartmann, A., 2007. Fourth International Workgroup on Genotoxicity testing: Results of the in vivo
746 Comet assay workgroup. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*
747 627, 31–35. <https://doi.org/10.1016/j.mrgentox.2006.08.011>

- Burns, J., Boogaard, H., Turley, R., Pfadenhauer, L.M., van Erp, A.M., Rohwer, A.C., Rehfuss, E., 2014. Interventions to reduce ambient particulate matter air pollution and their effect on health. *Cochrane Database of Systematic Reviews*. <https://doi.org/10.1002/14651858.CD010919>
- Cassoni, F., Bocchi, C., Martino, A., Pinto, G., Fontana, F., Buschini, A., 2004. The Salmonella mutagenicity of urban airborne particulate matter (PM_{2.5}) from eight sites of the Emilia-Romagna regional monitoring network (Italy). *Science of The Total Environment* 324, 79–90. <https://doi.org/10.1016/j.scitotenv.2003.10.030>
- Chen, S.-C., Tsai, C.-J., Huang, C.-Y., Chen, H.-D., Chen, S.-J., Lin, C.-C., Tsai, J.-H., Chou, C.C.-K., Lung, S.-C.C., Huang, W.-R., Roam, G.-D., Wu, W.-Y., Smolik, J., Dzumbova, L., 2010. Chemical Mass Closure and Chemical Characteristics of Ambient Ultrafine Particles and other PM Fractions. *Aerosol Science and Technology* 44, 713–723. <https://doi.org/10.1080/02786826.2010.486385>
- Cho, C.-C., Hsieh, W.-Y., Tsai, C.-H., Chen, C.-Y., Chang, H.-F., Lin, C.-S., 2018. In vitro and in vivo experimental studies of PM_{2.5} on disease progression. *International Journal of Environmental Research and Public Health* 15, 1380. <https://doi.org/10.3390/ijerph15071380>
- Clougherty, J.E., 2010. A growing role for gender analysis in air pollution epidemiology. *Environmental Health Perspectives* 118, 167–176. <https://doi.org/10.1289/ehp.0900994>
- Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. *Mutagenesis* 23, 143–151. <https://doi.org/10.1093/mutage/gem051>
- Costa, A.N., Moreno, V., Prieto, M.J., Urbano, A.M., Alpoim, M.C., 2010. Induction of morphological changes in BEAS-2B human bronchial epithelial cells following chronic sub-cytotoxic and mildly cytotoxic hexavalent chromium exposures. *Molecular Carcinogenesis* n/a-n/a. <https://doi.org/10.1002/mc.20624>
- Courcot, E., Leclerc, J., Lafitte, J.-J., Mensier, E., Jaillard, S., Gosset, P., Shirali, P., Pottier, N., Broly, F., Lo-Guidice, J.-M., 2012. Xenobiotic metabolism and disposition in human lung cell models: comparison with in vivo expression profiles. *Drug Metabolism and Disposition* 40, 1953–1965. <https://doi.org/10.1124/dmd.112.046896>
- Cury, P.M., Lichtenfels, A.J.F.C., Reymão, M.S.F., Conceição, G.M.S., Capelozi, V.L., Saldiva, P.H.N., 2000. Urban levels of air pollution modifies the progression of urethane-induced lung tumours in mice. *Pathology - Research and Practice* 196, 627–633. [https://doi.org/10.1016/S0344-0338\(00\)80006-0](https://doi.org/10.1016/S0344-0338(00)80006-0)
- Dusinska, M., Tulinska, J., El Yamani, N., Kuricova, M., Liskova, A., Rollerova, E., Rundén-Pran, E., Smolkova, B., 2017. Immunotoxicity, genotoxicity and epigenetic toxicity of nanomaterials: New strategies for toxicity testing? *Food and Chemical Toxicology* 109, 797–811. <https://doi.org/10.1016/j.fct.2017.08.030>
- Fenech, M., 2000. The in vitro micronucleus technique. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 455, 81–95. [https://doi.org/10.1016/S0027-5107\(00\)00065-8](https://doi.org/10.1016/S0027-5107(00)00065-8)
- Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 534, 65–75. [https://doi.org/10.1016/S1383-5718\(02\)00249-8](https://doi.org/10.1016/S1383-5718(02)00249-8)
- Ferkol, T., Schraufnagel, D., 2014. The Global Burden of Respiratory Disease. *Annals ATS* 11, 404–406. <https://doi.org/10.1513/AnnalsATS.201311-405PS>
- Fröhlich, E., Salar-Behzadi, S., 2014. Toxicological Assessment of Inhaled Nanoparticles: Role of in Vivo, ex Vivo, in Vitro, and in Silico Studies. *International Journal of Molecular Sciences* 15, 4795–4822. <https://doi.org/10.3390/ijms15034795>
- Gábelová, A., Valovicová, Z., Bacová, G., Lábaj, J., Binková, B., Topinka, J., Sevastyanova, O., Srám, R.J., Kalina, I., Habalová, V., Popov, T.A., Panev, T., Farmer, P.B., 2007a. Sensitivity of different endpoints for in vitro measurement of genotoxicity of extractable organic matter associated with ambient airborne particles (PM₁₀). *Mutat. Res.* 620, 103–113. <https://doi.org/10.1016/j.mrfmmm.2007.02.026>
- Gábelová, A., Valovičová, Z., Horváthová, E., Slameňová, D., Binková, B., Šrám, R.J., Farmer, P.B., 2004. Genotoxicity of environmental air pollution in three European cities: Prague, Košice and Sofia. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 563, 49–59. <https://doi.org/10.1016/j.mrgentox.2004.05.014>
- Gábelová, A., Valovicová, Z., Lábaj, J., Bacová, G., Binková, B., Farmer, P.B., 2007b. Assessment of oxidative DNA damage formation by organic complex mixtures from airborne particles PM₁₀. *Mutat. Res.* 620, 135–144. <https://doi.org/10.1016/j.mrfmmm.2007.03.003>

- Garcia-Canton, C., Minet, E., Anadon, A., Meredith, C., 2013. Metabolic characterization of cell systems used in in vitro toxicology testing: Lung cell system BEAS-2B as a working example. *Toxicology in Vitro* 27, 1719–1727. <https://doi.org/10.1016/j.tiv.2013.05.001>
- Gualtieri, M., Grollino, M.G., Consales, C., Costabile, F., Manigrasso, M., Avino, P., Aufderheide, M., Cordelli, E., Di Liberto, L., Petralia, E., Raschellà, G., Stracquadanio, M., Wiedensohler, A., Pacchierotti, F., Zanini, G., 2018. Is it the time to study air pollution effects under environmental conditions? A case study to support the shift of in vitro toxicology from the bench to the field. *Chemosphere* 207, 552–564. <https://doi.org/10.1016/j.chemosphere.2018.05.130>
- Guerreiro, C., González Ortiz, A., Leeuw, F. de, Viana, M., Colette, A., European Environment Agency, 2018. Air quality in Europe - 2018 report.
- Haniu, H., Saito, N., Matsuda, Y., Tsukahara, T., Maruyama, K., Usui, Y., Aoki, K., Takanashi, S., Kobayashi, S., Nomura, H., Okamoto, M., Shimizu, M., Kato, H., 2013. Culture medium type affects endocytosis of multi-walled carbon nanotubes in BEAS-2B cells and subsequent biological response. *Toxicology in Vitro* 27, 1679–1685. <https://doi.org/10.1016/j.tiv.2013.04.012>
- Harrison, R.M., Yin, J., 2000. Particulate matter in the atmosphere: which particle properties are important for its effects on health? *Science of The Total Environment* 249, 85–101. [https://doi.org/10.1016/S0048-9697\(99\)00513-6](https://doi.org/10.1016/S0048-9697(99)00513-6)
- International Agency for Research on Cancer, 2015. Outdoor air pollution. IARC monographs on the evaluation of carcinogenic risks to humans. Volume 109.
- Jensen, K.A., Kembouche, Y., Elzbieta, E., Jacobsen, N.R., Wallin, H., Guiot, C., Spalla, O., Witschger, O., 2011. Final Protocol for producing suitable manufactured nanomaterial exposure media. The generic NANOGENOTOX dispersion protocol.
- Kim, H.J., Choi, M.G., Park, M.K., Seo, Y.R., 2017. Predictive and prognostic biomarkers of respiratory diseases due to particulate matter exposure. *Journal of Cancer Prevention* 22, 6–15. <https://doi.org/10.15430/JCP.2017.22.1.6>
- Krishna, G., Nath, J., Soler, L., Ong, T., 1986. Comparative in vivo and in vitro genotoxicity studies of airborne particle extract in mice. *Mutation Research/Genetic Toxicology* 171, 157–163. [https://doi.org/10.1016/0165-1218\(86\)90049-2](https://doi.org/10.1016/0165-1218(86)90049-2)
- Lapôtre-Ledoux, B., Plouvier, S., Cariou, M., Billot-Grasset, A., Chatignoux, E., 2019. Estimations régionales et départementales d'incidence et de mortalité par cancers en France, 2007-2016. Hauts-de-France. Santé publique France.
- Leclercq, B., Happillon, M., Antherieu, S., Hardy, E.M., Alleman, L.Y., Grova, N., Perdrix, E., Appenzeller, B.M., Lo Guidice, J.-M., Coddeville, P., Garçon, G., 2016. Differential responses of healthy and chronic obstructive pulmonary diseased human bronchial epithelial cells repeatedly exposed to air pollution-derived PM₄. *Environmental Pollution* 218, 1074–1088. <https://doi.org/10.1016/j.envpol.2016.08.059>
- Leclercq, B., Platel, A., Antherieu, S., Alleman, L.Y., Hardy, E.M., Perdrix, E., Grova, N., Riffault, V., Appenzeller, B.M., Happillon, M., Nesslany, F., Coddeville, P., Lo-Guidice, J.-M., Garçon, G., 2017. Genetic and epigenetic alterations in normal and sensitive COPD-diseased human bronchial epithelial cells repeatedly exposed to air pollution-derived PM_{2.5}. *Environmental Pollution* 230, 163–177. <https://doi.org/10.1016/j.envpol.2017.06.028>
- Lelieveld, J., Klingmüller, K., Pozzer, A., Pöschl, U., Fnais, M., Daiber, A., Münzel, T., 2019. Cardiovascular disease burden from ambient air pollution in Europe reassessed using novel hazard ratio functions. *European Heart Journal*. <https://doi.org/10.1093/eurheartj/ehz135>
- Lemos, A.T., Lemos, C.T. de, Flores, A.N., Pantoja, E.O., Rocha, J.A.V., Vargas, V.M.F., 2016. Genotoxicity biomarkers for airborne particulate matter (PM_{2.5}) in an area under petrochemical influence. *Chemosphere* 159, 610–618. <https://doi.org/10.1016/j.chemosphere.2016.05.087>
- Lim, S.S., Vos, T., Flaxman, A.D., Danaei, G., Shibuya, K., Adair-Rohani, H., AlMazroa, M.A., Amann, M., Anderson, H.R., Andrews, K.G., Aryee, M., Atkinson, C., Bacchus, L.J., Bahalim, A.N., Balakrishnan, K., Balmes, J., Barker-Collo, S., Baxter, A., Bell, M.L., Blore, J.D., Blyth, F., Bonner, C., Borges, G., Bourne, R., Boussinesq, M., Brauer, M., Brooks, P., Bruce, N.G., Brunekreef, B., Bryan-Hancock, C., Bucello, C., Buchbinder, R., Bull, F., Burnett, R.T., Byers, T.E., Calabria, B., Carapetis, J., Carnahan, E., Chafe, Z., Charlson, F., Chen, H., Chen, J.S., Cheng, A.T.-A., Child, J.C., Cohen, A., Colson, K.E., Cowie, B.C., Darby, S., Darling, S., Davis, A., Degenhardt, L., Dentener, F., Des Jarlais, D.C., Devries, K., Dherani, M., Ding, E.L., Dorsey, E.R., Driscoll, T., Edmond, K., Ali, S.E., Engell, R.E., Erwin, P.J., Fahimi, S., Falder, G., Farzadfar, F., Ferrari, A., Finucane, M.M., Flaxman, S., Fowkes, F.G.R., Freedman, G., Freeman, M.K., Gakidou, E., Ghosh, S., Giovannucci, E., Gmel, G., Graham, K., Grainger, R., Grant, B., Gunnell, D., Gutierrez, H.R., Hall, W., Hoek, H.W., Hogan, A., Hosgood, H.D., Hoy, D., Hu, H., Hubbell, B.J., Hutchings, S.J., Ibeanusi, S.E., Jacklyn, G.L., Jasrasaria, R., Jonas, J.B., Kan, H., Kanis, J.A., Kassebaum,

- N., Kawakami, N., Khang, Y.-H., Khatibzadeh, S., Khoo, J.-P., Kok, C., Laden, F., Lalloo, R., Lan, Q., Lathlean, T., Leasher, J.L., Leigh, J., Li, Y., Lin, J.K., Lipshultz, S.E., London, S., Lozano, R., Lu, Y., Mak, J., Malekzadeh, R., Mallinger, L., Marcenes, W., March, L., Marks, R., Martin, R., McGale, P., McGrath, J., Mehta, S., Memish, Z.A., Mensah, G.A., Merriman, T.R., Micha, R., Michaud, C., Mishra, V., Hanafiah, K.M., Mokdad, A.A., Morawska, L., Mozaffarian, D., Murphy, T., Naghavi, M., Neal, B., Nelson, P.K., Nolla, J.M., Norman, R., Olives, C., Omer, S.B., Orchard, J., Osborne, R., Ostro, B., Page, A., Pandey, K.D., Parry, C.D., Passmore, E., Patra, J., Pearce, N., Pelizzari, P.M., Petzold, M., Phillips, M.R., Pope, D., Pope, C.A., Powles, J., Rao, M., Razavi, H., Rehfuess, E.A., Rehm, J.T., Ritz, B., Rivara, F.P., Roberts, T., Robinson, C., Rodriguez-Portales, J.A., Romieu, I., Room, R., Rosenfeld, L.C., Roy, A., Rushton, L., Salomon, J.A., Sampson, U., Sanchez-Riera, L., Sanman, E., Sapkota, A., Seedat, S., Shi, P., Shield, K., Shivakoti, R., Singh, G.M., Sleet, D.A., Smith, E., Smith, K.R., Stapelberg, N.J., Steenland, K., Stöckl, H., Stovner, L.J., Straif, K., Straney, L., Thurston, G.D., Tran, J.H., Van Dingenen, R., van Donkelaar, A., Veerman, J.L., Vijayakumar, L., Weintraub, R., Weissman, M.M., White, R.A., Whiteford, H., Wiersma, S.T., Wilkinson, J.D., Williams, H.C., Williams, W., Wilson, N., Woolf, A.D., Yip, P., Zielinski, J.M., Lopez, A.D., Murray, C.J., Ezzati, M., 2012. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet* 380, 2224–2260. [https://doi.org/10.1016/S0140-6736\(12\)61766-8](https://doi.org/10.1016/S0140-6736(12)61766-8)
- Loomis, D., Grosse, Y., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Baan, R., Mattock, H., Straif, K., International Agency for Research on Cancer Monograph Working Group IARC, 2013. The carcinogenicity of outdoor air pollution. *Lancet Oncol.* 14, 1262–1263.
- Lovell, D.P., Omori, T., 2008. Statistical issues in the use of the comet assay. *Mutagenesis* 23, 171–182. <https://doi.org/10.1093/mutage/gen015>
- Magdolenova, Z., Collins, A., Kumar, A., Dhawan, A., Stone, V., Dusinska, M., 2014. Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. *Nanotoxicology* 8, 233–278. <https://doi.org/10.3109/17435390.2013.773464>
- Marabini, L., Ozgen, S., Turacchi, S., Aminti, S., Arnaboldi, F., Lonati, G., Fermo, P., Corbella, L., Valli, G., Bernardoni, V., Dell'Acqua, M., Vecchi, R., Becagli, S., Caruso, D., Corrado, G.L., Marinovich, M., 2017. Ultrafine particles (UFPs) from domestic wood stoves: genotoxicity in human lung carcinoma A549 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 820, 39–46. <https://doi.org/10.1016/j.mrgentox.2017.06.001>
- Meuwissen, R., 2005. Mouse models for human lung cancer. *Genes & Development* 19, 643–664. <https://doi.org/10.1101/gad.1284505>
- Mutlu, G.M., Green, D., Bellmeyer, A., Baker, C.M., Burgess, Z., Rajamannan, N., Christman, J.W., Foiles, N., Kamp, D.W., Ghio, A.J., Chandel, N.S., Dean, D.A., Sznajder, J.I., Budinger, G.R.S., 2007. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J. Clin. Invest.* 117, 2952–2961. <https://doi.org/10.1172/JCI30639>
- Nabizadeh, R., Yousefian, F., Moghadam, V.K., Hadei, M., 2019. Characteristics of cohort studies of long-term exposure to PM_{2.5}: a systematic review. *Environmental Science and Pollution Research*. <https://doi.org/10.1007/s11356-019-06382-6>
- Nemmar, A., Holme, J.A., Rosas, I., Schwarze, P.E., Alfaro-Moreno, E., 2013. Recent advances in particulate matter and nanoparticle toxicology: A review of the in vivo and in vitro studies. *BioMed Research International* 2013, 1–22. <https://doi.org/10.1155/2013/279371>
- OECD, 2016a. OECD test guideline No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4. OECD. <https://doi.org/10.1787/9789264224438-en>
- OECD, 2016b. OECD test guideline No. 489: In vivo mammalian alkaline comet assay, OECD Guidelines for the Testing of Chemicals, Section 4. OECD. <https://doi.org/10.1787/9789264264885-en>
- OECD, 2016c. OECD test guideline No. 474: Mammalian erythrocyte micronucleus test, OECD Guidelines for the Testing of Chemicals, Section 4. OECD. <https://doi.org/10.1787/9789264264762-en>
- Oh, S.M., Kim, H.R., Park, Y.J., Lee, S.Y., Chung, K.H., 2011. Organic extracts of urban air pollution particulate matter (PM_{2.5})-induced genotoxicity and oxidative stress in human lung bronchial epithelial cells (BEAS-2B cells). *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 723, 142–151. <https://doi.org/10.1016/j.mrgentox.2011.04.003>
- Pereira, T.S., Beltrami, L.S., Rocha, J.A.V., Broto, F.P., Comellas, L.R., Salvadori, D.M.F., Vargas, V.M.F., 2013. Toxicogenetic monitoring in urban cities exposed to different airborne contaminants. *Ecotoxicology and Environmental Safety* 90, 174–182. <https://doi.org/10.1016/j.ecoenv.2012.12.029>

- Peters, A., Veronesi, B., Calderón-Garcidueñas, L., Gehr, P., Chen, L.C., Geiser, M., Reed, W., Rothen-Rutishauser, B., Schürch, S., Schulz, H., 2006. Translocation and potential neurological effects of fine and ultrafine particles a critical update. Part Fibre Toxicol 3, 1–13. <https://doi.org/10.1186/1743-8977-3-13>
- Rayner, R.E., Makena, P., Prasad, G.L., Cormet-Boyaka, E., 2019. Optimization of Normal Human Bronchial Epithelial (NHBE) Cell 3D Cultures for in vitro Lung Model Studies. Sci Rep 9, 500. <https://doi.org/10.1038/s41598-018-36735-z>
- Reymão, M.S.F., Cury, P.M., Lichtenfels, A.J.F.C., Lemos, M., Battlehner, C.N., Conceição, G.M.S., Capelozzi, V.L., Montes, G.S., Júnior, M.F., Martins, M.A., Böhm, G.M., Saldiva, P.H.N., 1997. Urban Air Pollution Enhances the Formation of Urethane-Induced Lung Tumors in Mice. Environmental Research 74, 150–158. <https://doi.org/10.1006/enrs.1997.3740>
- Rückerl, R., Schneider, A., Breitner, S., Cyrus, J., Peters, A., 2011. Health effects of particulate air pollution: A review of epidemiological evidence. Inhalation Toxicology 23, 555–592. <https://doi.org/10.3109/08958378.2011.593587>
- Saleh, Y., Anthérieu, S., Dusautoir, R., Alleman, L., Sotty, J., De Sousa, C., Platel, A., Perdrix, E., Riffault, V., Fronval, I., Nesslany, F., Canivet, L., Lo-Guidice, J., 2019. Exposure to Atmospheric Ultrafine Particles Induces Severe Lung Inflammatory Response and Tissue Remodeling in Mice. Int. J. Environ. Res. Public Health Accepted.
- Schraufnagel, D.E., Balmes, J.R., Cowl, C.T., De Matteis, S., Jung, S.-H., Mortimer, K., Perez-Padilla, R., Rice, M.B., Riojas-Rodriguez, H., Sood, A., Thurston, G.D., To, T., Vanker, A., Wuebbles, D.J., 2019. Air Pollution and Noncommunicable Diseases. Chest 155, 417–426. <https://doi.org/10.1016/j.chest.2018.10.041>
- Shamsipour, M., Hassanvand, M.S., Gohari, K., Yunesian, M., Fotouhi, A., Naddafi, K., Sheidaei, A., Faridi, S., Akhlaghi, A.A., Rabiei, K., Mehdipour, P., Mahdavi, M., Amini, H., Farzadfar, F., 2019. National and sub-national exposure to ambient fine particulate matter (PM_{2.5}) and its attributable burden of disease in Iran from 1990 to 2016. Environmental Pollution 255, 113173. <https://doi.org/10.1016/j.envpol.2019.113173>
- Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175, 184–191. [https://doi.org/10.1016/0014-4827\(88\)90265-0](https://doi.org/10.1016/0014-4827(88)90265-0)
- Sotty, J., Garçon, G., Denayer, F.-O., Alleman, L.-Y., Saleh, Y., Perdrix, E., Riffault, V., Dubot, P., Lo-Guidice, J.-M., Canivet, L., 2019. Toxicological effects of ambient fine (PM_{2.5-0.18}) and ultrafine (PM_{0.18}) particles in healthy and diseased 3D organo-typic mucociliary-phenotype models. Environmental Research 108538. <https://doi.org/10.1016/j.envres.2019.108538>
- Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., Sasaki, Y.F., 2000. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis 35, 206–221. [https://doi.org/10.1002/\(SICI\)1098-2280\(2000\)35:3<206::AID-EM8>3.0.CO;2-J](https://doi.org/10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J)
- Topinka, J., Schwarz, L.R., Wiebel, F.J., Cerná, M., Wolff, T., 2000. Genotoxicity of urban air pollutants in the Czech Republic. Part II. DNA adduct formation in mammalian cells by extractable organic matter. Mutat. Res. 469, 83–93.
- Weber, S.A., Insaf, T.Z., Hall, E.S., Talbot, T.O., Huff, A.K., 2016. Assessing the impact of fine particulate matter (PM_{2.5}) on respiratory-cardiovascular chronic diseases in the New York City Metropolitan area using Hierarchical Bayesian Model estimates. Environmental Research 151, 399–409. <https://doi.org/10.1016/j.envres.2016.07.012>
- Witte, I., Plappert, U., de Wall, H., Hartmann, A., 2007. Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation Part III: The Comet Assay as an Alternative to In Vitro Clastogenicity Tests for Early Drug Candidate Selection. Toxicological Sciences 97, 21–26. <https://doi.org/10.1093/toxsci/kfl192>
- World Health Organization, 2019. World Health Organization. Global Health Observatory.
- World Health Organization, 2018. Global Health Estimates 2016: Deaths by cause, age, sex, by country and by region, 2000–2016. Geneva: World Health Organization, 2018.
- World Health Organization, 2016. Ambient air pollution: A global assessment of exposure and burden of disease.
- Xia, T., Zhu, Y., Mu, L., Zhang, Z.-F., Liu, S., 2016. Pulmonary diseases induced by ambient ultrafine and engineered nanoparticles in twenty-first century. Nat. Sci. Rev. nww064. <https://doi.org/10.1093/nsr/nww064>
- Xing, Y.-F., Xu, Y.-H., Shi, M.-H., Lian, Y.-X., 2016. The impact of PM_{2.5} on the human respiratory system. J Thorac Dis 8, E69-74. <https://doi.org/10.3978/j.issn.2072-1439.2016.01.19>

986 Yitshak-Sade, M., Kloog, I., Zanobetti, A., Schwartz, J.D., 2019. Estimating the causal effect of annual
987 PM2.5 exposure on mortality rates in the Northeastern and mid-Atlantic states. Environmental
988 Epidemiology 3, e052. <https://doi.org/10.1097/EE9.0000000000000052>
989
990

991

992

TABLE AND FIGURE LEGENDS

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 µg PM/cm² 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 µg/cm²). * p<0.05 (Mann-Whitney U-test). One experiment, 2 biological replicates, 4 technical replicates.

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

Chromosomal aberrations were determined with the micronucleus test after 24 h exposure to 5 µg PM/cm² 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/cm²). * p<0.05 (CHI-squared test). One experiment, 2 biological replicates, 4 technical replicates.

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 µg of particles. The control group received 37 µL 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. Results of *in vivo* genotoxicity/mutagenicity assessment after subacute exposure of 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 10⁶ 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 10⁶ 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/cm² 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.**

1060 Cells were treated with 5 µg/cm² of airborne FPs and UFPs. Comet assay was performed immediately
1061 after 4h exposure at the ALI whereas micronucleus test was performed after 24h exposure in submerged
1062 conditions followed by a 2 days (BEAS-2B and NCI-H292 cells) or a 5 days (NHBE cells) recovery period.
1063 -: negative result; +: positive result.

1064

1065 **Table 5. Summary of in vivo genotoxicity/mutagenicity tests results.**

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

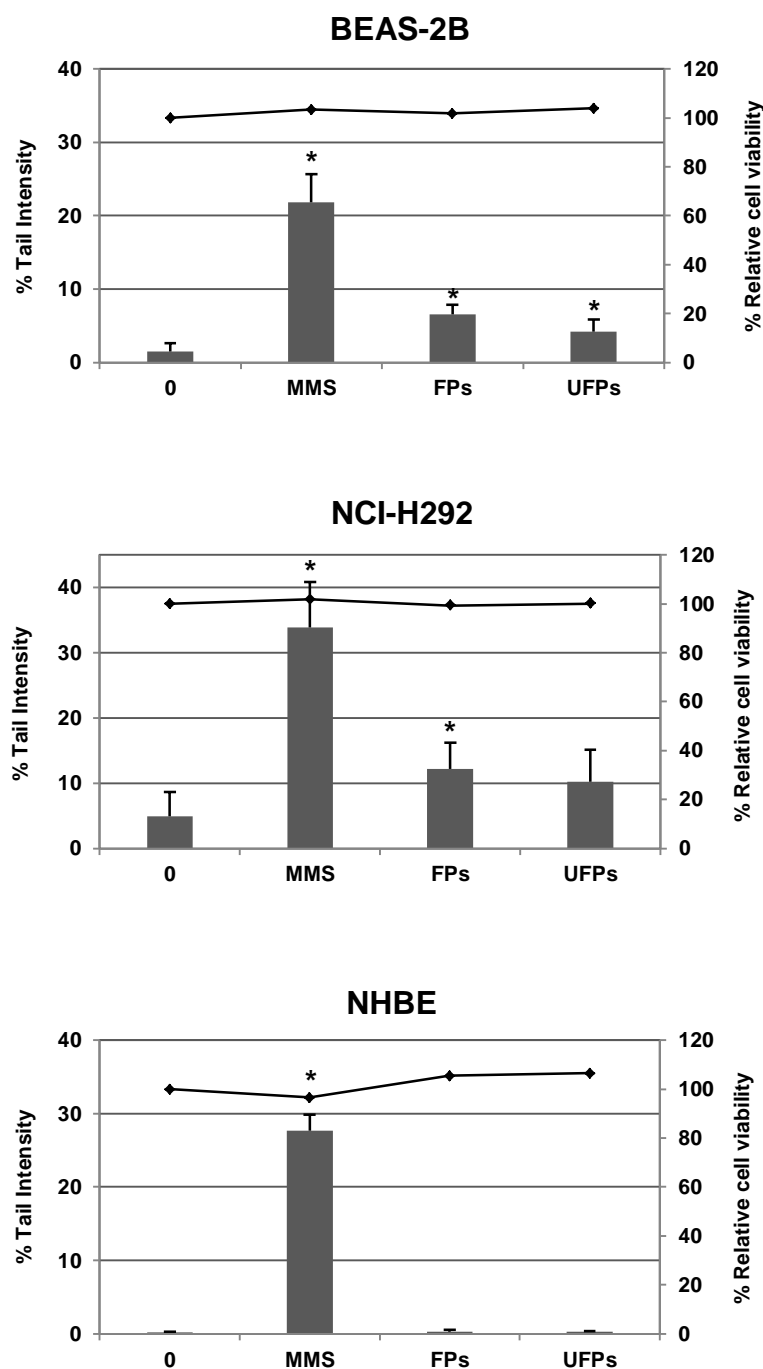


Figure 1. Results of *in vitro* comet assays.

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

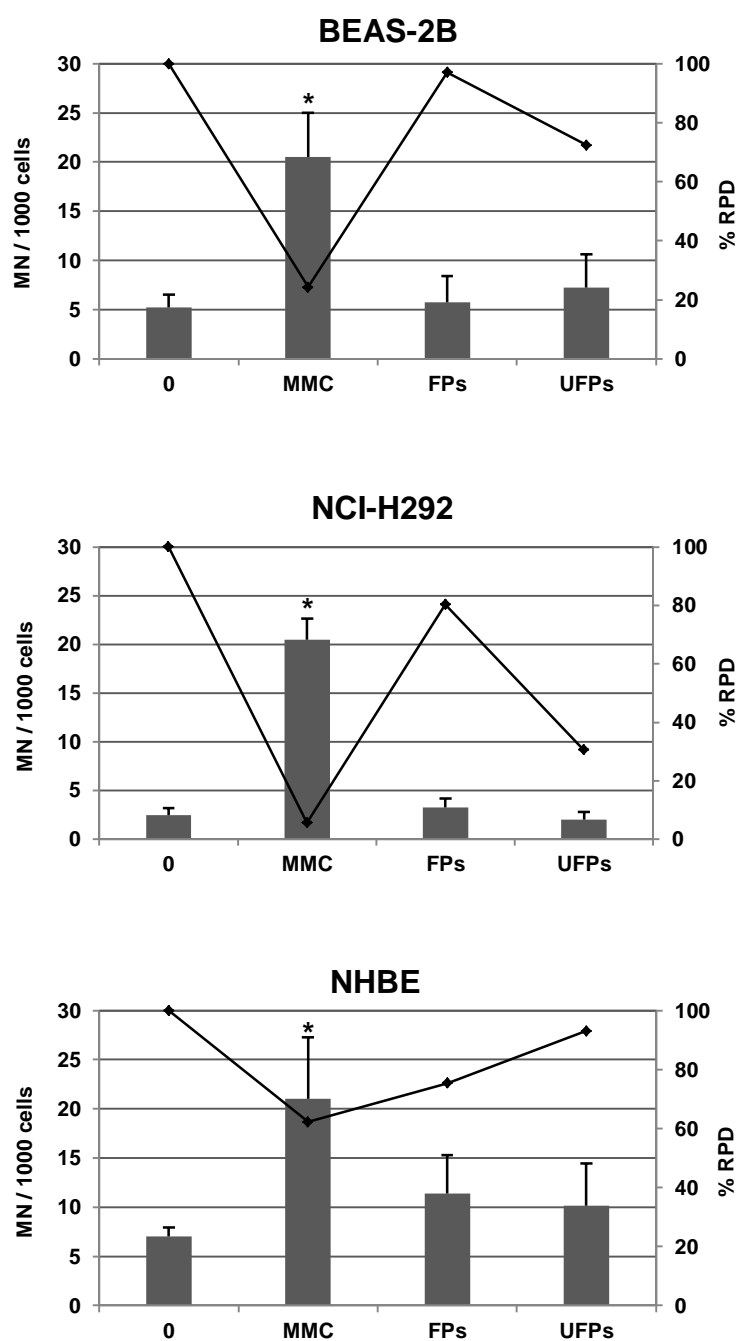


Figure 2. Results of *in vitro* micronucleus tests.

Chromosomal aberrations were determined with the micronucleus test after 24 h exposure to 5 $\mu\text{g PM}/\text{cm}^2$ 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 $\mu\text{g}/\text{cm}^2$). * $p < 0.05$ (CHI-squared test). One experiment, 2 biological replicates, 4 technical replicates.

FIGURE 3

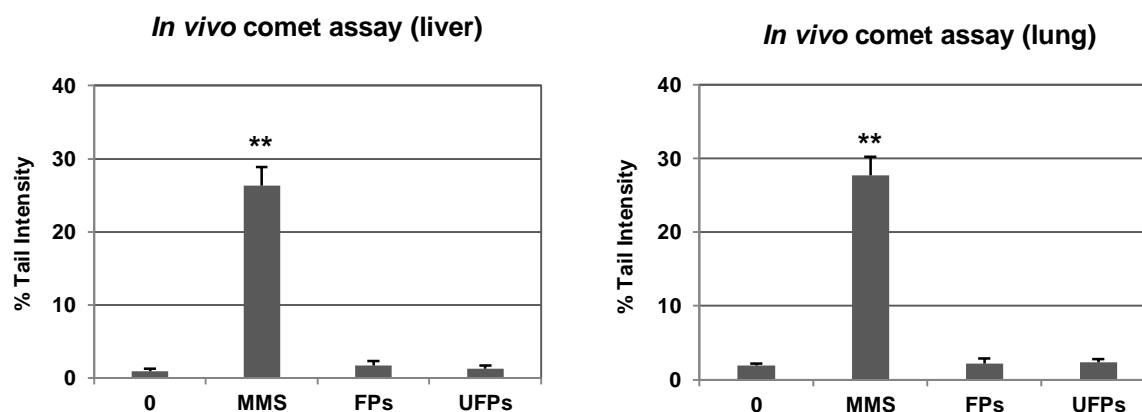


Figure 3. Results of *in vivo* genotoxicity assessment after acute exposure of airborne particles in mice.

Animals (n=5) received 2 treatments at 24-hour interval at the dose of 100 μ g of particles. The control group received 37 μ 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

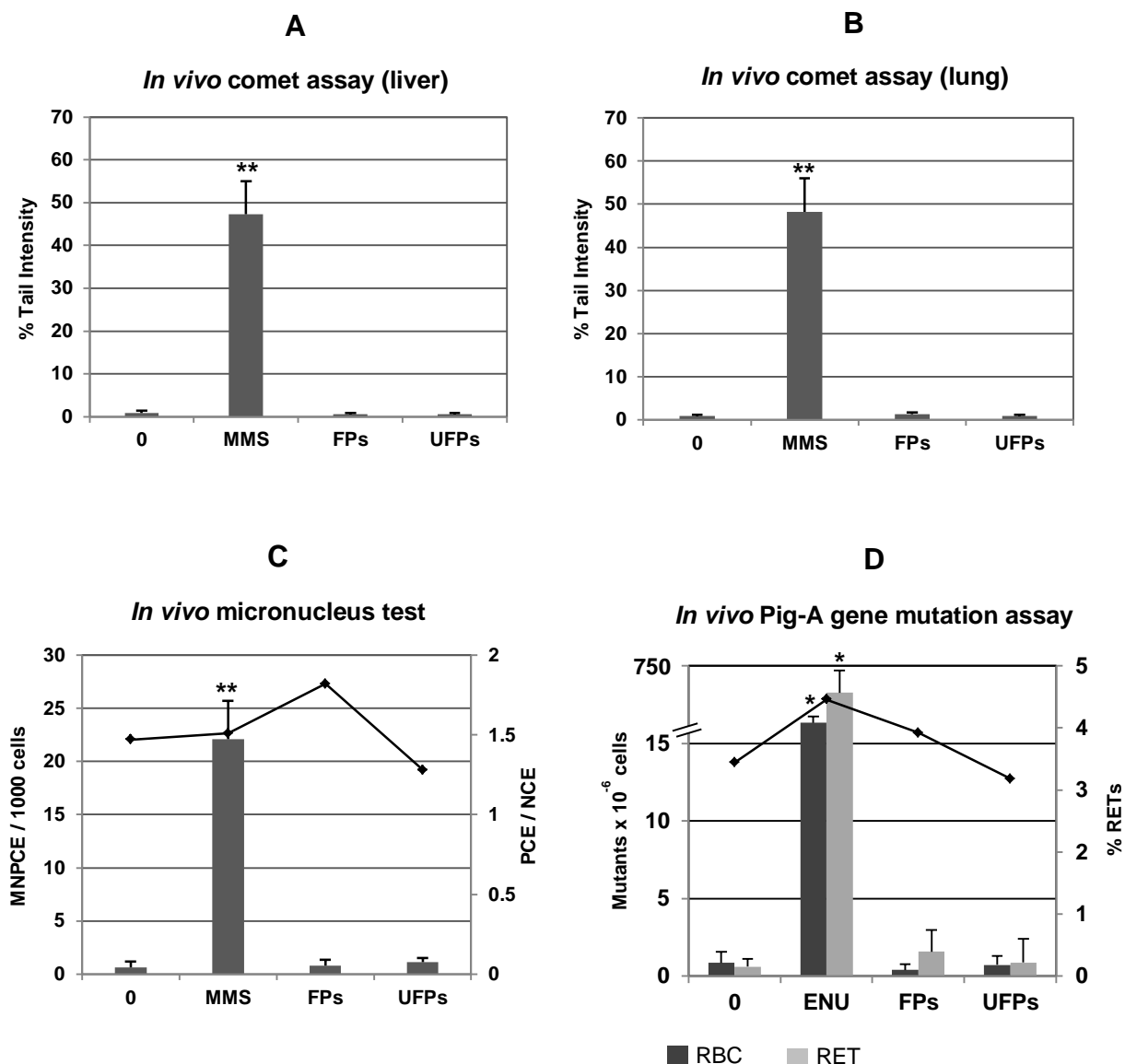


Figure 4. Results of *in vivo* genotoxicity/mutagenicity assessment after subacute exposure of airborne particles in mice.

Animals (n=5) received 10 μ g of particles, 3 times a week, during 1 month. The control group received 37 μ L 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 10⁶ 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

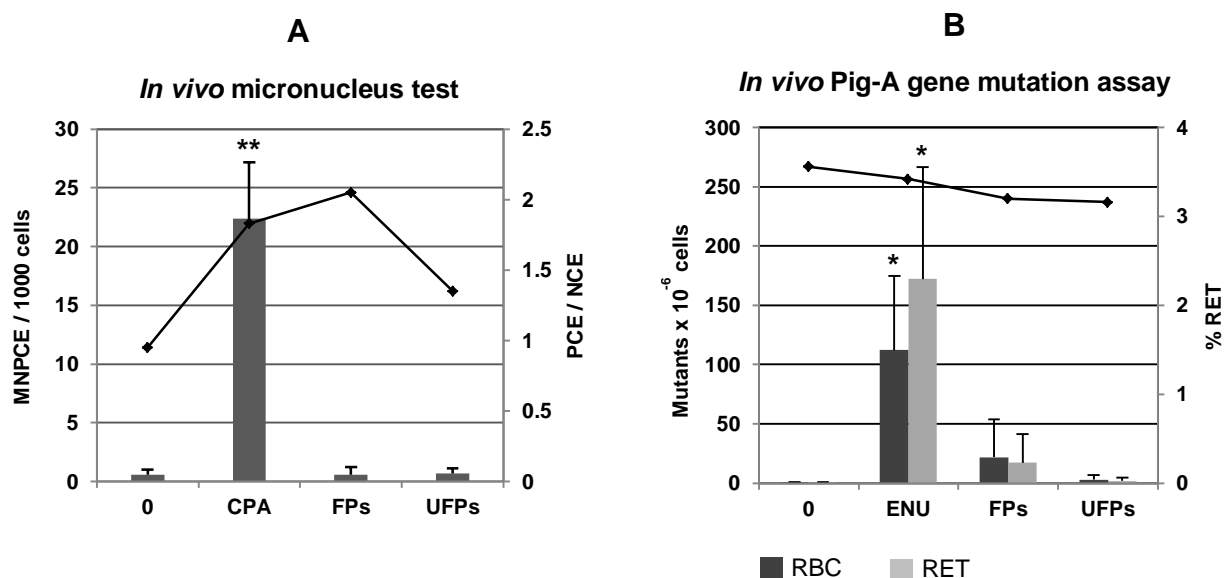


Figure 5. Results of *in vivo* genotoxicity/mutagenicity assessment after subchronic exposure of airborne particles in mice.

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 10⁶ 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
Comet assay	Primary DNA damage	ALI	4 hours	X	X	X
Micronucleus test	Chromosomal aberrations	Immersion	24 hours	X	X	X

Cells were treated with 5 µg PM/cm² 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.

SPECIFICATIONS	RESULTS
Donor characteristics	
Age (years)	49
Sex	Female
Race	Caucasian
Smoking habits	Non-smoker
Alcohol use	Non-alcohol user
Virus Testing	
HIV Test	Not detected
HBV Test	Not detected
HCV Test	Not detected
Microbial Testing	
Sterility Test	Negative
Mycoplasma	Negative
Cell Performance Testing	
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
Comet assay	Primary DNA damage	Liver, lung	X	X	
Micronucleus test	Chromosomal aberrations	Bone marrow		X	X
Pig-A gene mutation assay	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
Comet assay	Primary DNA damage	ALI	-	+	+
Micronucleus test	Chromosomal aberrations	Immersion	-	-	-

Cells were treated with 5 µg/cm² 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
Comet assay	Primary DNA damage	Liver, lung	-	-	n.a.
Micronucleus test	Chromosomal aberrations	Bone marrow	n.a.	-	-
Pig-A gene mutation assay	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.