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On the dose-response association of fine and ultrafine particles in an urban atmosphere: toxicological outcomes on bronchial cells at realistic doses of exposure at the Air Liquid Interface

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Abbreviations: **AMS**, Aerosol Mass Spectrometer; **ALI**, Air-liquid interface; **ARE**, antioxidant responsive element; ***ATM***, Ataxia telangiectasia mutated; **Comet_-total**, Comet assay, tail moment; **Comet_FPG**, Comet assay with formamidopyrimidine [fapy]-DNA glycosylase (FPG)enzyme, tail moment; **Comet_ENDO_III**, Comet assay with endonuclease III (ENDO III) enzyme, tail moment; **Comet_ T4PDG**, Comet assay with T4 endonuclease V (T4PDG), tail moment; **CS**, condensation sink; ***Cxcl-8***, C-X-C Motif Chemokine Ligand 8; **f_NH4**, fractional mass of ammonia; **f_BBOA**, fractional mass of Biomass-Burning Organic Aerosol, a component of organic aerosol identified by AMS-PMF and related to biomass combustion (during winter mainly from domestic heating); **f_BC**, fractional mass of black carbon BC; **f_HOA**, fractional mass of Hydrocarbon-like Organic Aerosol, an OA component associated with traffic/fossil-fuels combustion identified by AMS-PMF; **f_NO3**, fractional of nitrate; **f_OOA**, fractional mass of Oxidized Organic Aerosols, components of organic aerosol identified by AMS-PMF and attributed to secondary formation and/or ageing of the particles; **f_Org**, fractional mass of organic aerosol; **f_SO4**, fraction mass of sulphates; ***Gadd45α***, Growth Arrest and DNA-damage-inducible, alpha; ***HMOX***, Heme Oxygenase gene; ***NQO1***, NAD(P)H Quinone Dehydrogenase 1; **NAM**, New Approach Methodologies; OA, Organic aerosol; **PM**, Particulate Matter; **UFP or $PM_{0.1}$**, Ultrafine particles; **PM_1**, PM with median aerodynamic diameter lower than 1 μm; **RHAPS**, Redox-activity and Health-effects of Atmospheric Primary and Secondary aerosol project; **SMPS**, Scanning Mobility Particle Sizer; **m_PM_1**, Theoretical mass of particles deposited on the lung cells; **N_PM_1**, Theoretical number of particles deposited on the lung cells; **sa_PM_1**, Theoretical particles' surface area deposited on the lung cells; **m_UFP**, Theoretical mass of ultrafine particles deposited on the lung cells; **N_UFP**, Theoretical number of ultrafine particles deposited on the lung cells; **sa_UFP**, Theoretical ultrafine particles' surface area deposited on the lung cells; **f_unex**, unexplained fractional mass.

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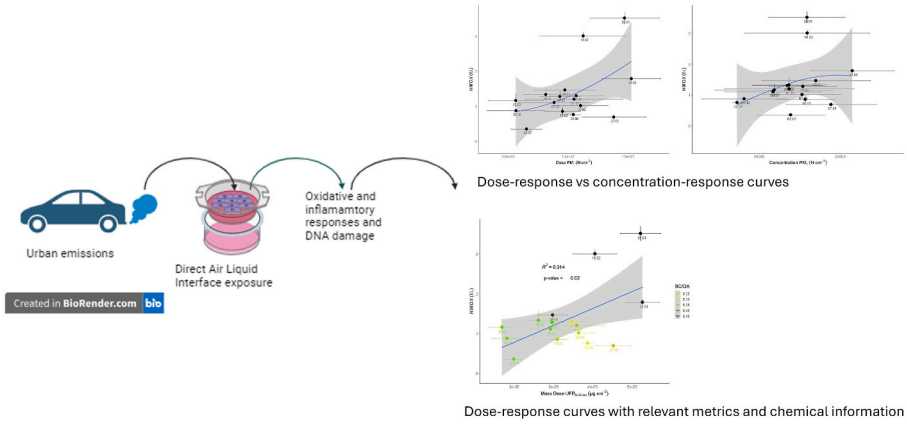
HIGHLIGHTS

- Oxidative responses are induced by freshly emitted combustion particles.
- DNA damage responses are associated to organic aerosol species.
- To be protective for health mass or number metrics must be associated with composition/aging information.
- The black carbon to organic carbon ratio is a good proxy for composition/aging and relevant to understanding PM toxicology.
- Effects reported as dose-response curves may differ from concentration-response ones.

GRAPHICAL ABSTRACT



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ABSTRACT

Air pollution and particulate matter (PM) are the leading environmental cause of death worldwide. Exposure limits have lowered to increase the protection of human health; accordingly, it becomes increasingly important to understand the toxicological mechanisms on cellular models at low airborne PM concentrations which are relevant for actual human exposure. The use of air liquid interface (ALI) models, which mimic the interaction between airborne pollutants and lung epithelia, is also gaining importance in inhalation toxicological studies. This study reports the effects of ALI direct exposure of bronchial epithelial cells BEAS-2B to ambient PM_1 (i.e. particles with aerodynamic diameter lower than 1 μm). Gene expression (HMOX, Cxcl-8, ATM, Gadd45-a and NQO1), interleukin (IL)-8 release, and DNA damage (Comet assay) were evaluated after 24 h of exposure. We report the dose-response curves of the selected toxicological outcomes, together with the concentration-response association and we show that the two curves differ for specific responses highlighting that concentration-response association may be not relevant for understanding toxicological outcomes. Noteworthy, we show that pro-oxidant effects may be driven by the deposition of freshly emitted particles, regardless of the airborne PM_1 mass concentration. Furthermore, we show that reference airborne PM_1 metrics, namely airborne mass concentration, may not always reflect the toxicological process triggered by the aerosol.

These findings underscore the importance of considering different aerosol metrics to assess the toxicological potency of fine and ultrafine particles. To better protect human health additional metrics should be defined, than account for the properties of the entire aerosol mixture including specific as particle size (i.e. particles with aerodynamic diameter lower than 20 nm), the relevant aerosol sources (e.g., traffic combustion, secondary organic aerosol …) as well as their atmospheric processing (freshly emitted vs aged ones).

1. Introduction

Air pollution and airborne particulate matter (PM) are among the first causes of non-communicable disease death in the world (Cohen et al., 2017; Ostro et al., 2018; Bennitt et al., 2021). Despite the strong epidemiological evidence linking air pollution and health outcomes, such as cardiopulmonary diseases and lung cancer (Raaschou-Nielsen et al., 2013; Renzi et al., 2017; HEI Panel on the Health, 2022; Chen et al., 2023; Manisalidis et al., 2020), recent findings show that airborne $PM_{2.5}$ can have significant health effects even at low and "safe" concentrations (Weichenthal et al., 2022). This raises critical questions about the mode(s) of action of $PM_{2.5}$ at low concentrations and which specific particulate components trigger peculiar lung cells responses.

Toxicological studies have long identified oxidative stress and inflammatory responses as crucial drivers of the adverse biological effects induced by airborne PM (Macnee, 2001; Campbell et al., 2021; Møller et al., 2014; Jia et al., 2021; Lee et al., 2014; Costa et al., 2020). However, most of the toxicological results available are based on PM exposure concentrations orders of magnitude higher those typically encountered in real life environment. In fact, modelling data starting from outdoor PM concentrations report lung deposited mass of fine and coarse particles in the order of few micrograms that translates in fraction of nanograms per square centimetre of the lungs epithelial surface (Segal et al., 2002; Hofmann, 2011; Koullapis et al., 2018; Manigrasso et al., 2020; Avino et al., 2016). Additionally, new approach methodologies (NAMs) (Ramanarayanan et al., 2022; Escher et al., 2022; Schmeisser et al., 2023) is providing additional strategies to better assess the hazard and provide data for the risk assessment of PM by proper focusing on human-specific responses and by analysing molecular mechanism and kinetics.

In this perspective, recent advances in toxicological approaches provide new opportunities to improve our knowledge aimed at the evaluation of the hazard of particulates after inhalation. First, new exposure systems allow now to mimic the actual exposure at the air to membrane interface of lung epithelial cells (Buckley et al., 2024; Hakkarainen et al., 2023; Bannuscher et al., 2022) mimicking the actual human exposure. Second, novel *in vitro* models of the lung epithelium, although not yet standardize and with some limitations, offer more representative insight into lung epithelia (Selo et al., 2021; Xu et al., 2020; Rothen-Rutishauser et al., 2023; Braakhuis et al., 2023). Finally, the physical and chemical complexity of airborne $PM_{2.5}$ requires the comprehension of how the different group of chemicals may affect lung

cells. For example, PAHs are well known carcinogenic (Sarigiannis et al., 2015) substances and they are found associated with PM particles in different fractions. However, from a health perspective, identifying high-impact PM sources may be more effective for risk management, This, in fact, may help in prioritizing the efforts by reducing the emissions of sources with higher impact on human health. Providing evidence that specific air quality determinants have the potential to impact human health may pave the way for a better global health (Gao, 2021).

To improve the health of the populations living in urban areas, it is becoming more and more necessary to produce dose-response associations at low PM mass concentrations, Such curves, still missing, could help explaining the epidemiological association between low PM mass concentration of air pollution (Chen et al., 2023; Weichenthal et al., 2022) and adverse human responses. Accordingly, it has been reported the need of considering the composition of PM rather than the classical mass concentration metric in future epidemiological studies (Weichenthal et al., 2024).

It has been recently reported (Costabile et al., 2023) that exposure to traffic-related nanoparticles at low concentrations of PM_1 may have a major impact on an *in vitro* model of the lung epithelium. The experimental approach of our study allowed the understanding of how pro-oxidative aerosol properties vary according to particles aging and condensation. We explored the potential effects of doses of exposure, relevant for human exposure, on BEAS-2B cells, cultured and exposed at the air liquid interface (ALI), considering different toxicological endpoints. We analysed the gene expression related to inflammation (*Cxcl-8*), oxidative stress (*Hmox-1* and *NQO1*) and DNA damage (*ATM* and *Gadd45-α*), the release of the pro-inflammatory interleukin-8 (IL-8) and the DNA damage *via* the Comet assay. The results were then correlated with doses of exposure (in mass, number or surface area) of total PM_1, $PM_{0.1}$ (or UFP, i.e., particles with aerodynamic diameter lower than 100 nm) as well as selected size ranges previously associated to different PM properties and sources (Costabile et al., 2023). The size-related analysis provides valuable information about the potential health risk for exposed populations. The study represents a significant step in linking a new approach methodology, consisting in direct exposure of a lung epithelial model to ambient PM_1, to air pollution hazard characterization and next generation risk assessment. Notably we report here dose-response curves that can be useful to understanding the impact of fine and ultrafine PM on human health also at low $PM_{2.5}$ ambient concentrations commonly observed in urban areas of developed countries.

2. Material and method

2.1. Cell culture and exposure

Detailed description of the exposure procedure is reported in Costabile et al. (2023) (Costabile et al., 2023). Briefly, human bronchial epithelial cells BEAS-2B (#95102433, ECACC, Salisbury, UK) was maintained in LHC-9 medium in a 95% humidified incubator at 37 °C with 5% of CO_2, split every three days. Mycoplasma absence was routinely checked. 72 h before exposure BEAS-2B were seeded on collagen coated inserts (12 wells multiplate Teflon transwell inserts with 0.4 μm pores, collagen coated Corning, NY, USA) at a density of 40×10^3 cells/insert and let to grow. The inserts were then transferred to the site of exposure and 24 h before starting the first experiment, the medium at the apical side of each transwell was removed to let the cells differentiate at the air liquid interface. During each exposure six inserts were transferred into the CULTEX® RFS Compact module and the basal side of each chamber was filled with 4 mL of LHC-9 medium (Gibco, Life Technologies, Monza, Italy). Cells were exposed in ALI to native atmosphere (with a cutting cyclone to 1 μm of aerodynamic diameter) or to filtered air at 5 mL/min for 24 h from 8 a.m. to 7:59 a.m. of the subsequent day. Four different weeks of exposure were selected, three in winter (25th – 31st of January, 1st – 7th and 15th – February 21, 2021 and 28th of June – 4th of July 2021). During each week of exposure four independent experiments were performed (Table 1) and three additional inserts were kept in the local incubator and considered as reference for the biological response of cell cultured at ALI, but not exposed to airborne pollutants. Exposure doses were calculated, considering the aerodynamic diameter of particles, according to Aufderheide and colleagues (Aufderheide et al., 2013, 2017) by applying the physical laws describing the random deposition for Brownian movements of particles and the gravitational settling, of primary importance for particles with diameter higher than 300 nm. Deposition doses, expressed as number, or mass or surface area of deposited particles, were calculated primarily for PM_1 and ultrafine particles (UFP). To specifically investigate the toxicological role of specific size fractions, other than the PM_1 and UFP ones, we grouped the exposure data using the following additional ranges: the size range “8–20 nm” is considered for evaluating the impact of the exposure to the tiniest ultrafine particles, the size range “20–100 nm” is selected as representative of the remaining UFP fraction, the size range “100–300” nm, i.e., the particles between 100 nm and 300 nm, that represent the first part of the coagulation mode of the aerosol; the “300–500” nm size range is selected as representative of the group of particle with reduced deposition, driven mainly by gravimetric settling, while “500–1000” nm is representative of all the remaining particles with diameter bigger that 500 nm, which deposition determine mostly the deposited mass.

2.2. Aerosol characterization

Aerosol physicochemical properties characterization is reported in detail in Costabile et al. (2023). Briefly, state-of-the-art equipment, namely the aerosol mass spectrometer (AMS), the aethalometer, the scanning mobility particle sizer (SMPS), were used to characterise, with time resolution of minutes, the physico and chemical properties of PM_1 over the exposure periods. For details on the materials and methods and main results on the chemical and physical aerosol properties please refer to the above-mentioned article. For details on the organic aerosol (OA) source apportionment refer to the open dataset available at https://doi.org/10.5281/zenodo.11191226 (Paglione, M., and Rinaldi, M. 2024) “Atmospheric aerosol chemical characterization and organic aerosol source apportionment by HR-TOF-AMS in the Po Valley during RHAPS (2021) [Data set]”. Zenodo. https://doi.org/10.5281/zenodo.11191226). We focused here on PM_1 under dry conditions, but measurements of $PM_{2.5}$ are available and reported in Costabile et al. (2023). In fact, $PM_{2.5}$ and PM_1 measurements show ubiquitously tight correlations ($r^2 > 0.7$) although the $PM_1/PM_{2.5}$ ratios can vary, from ~0.7 to 1.2, depending on chemical environments, water contents and instrument uncertainties (Sun et al., 2020; Budisulistiorini et al., 2014). The correlation between airborne particles physical properties (considering number or mass of PM_1 or selected fractions of PM_1) and their chemical properties is already explored in Costabile et al. (2023) and here not further discussed.

2.3. DNA damage

Just after the end of the exposure one random insert among the air pollution exposed and one random insert among the filtered air exposed were selected. Inserts were transferred in a sterile multiwell plate, and cells were washed with 300 μL of sterile PBS. Cells were then detached with 250 μL of sterile trypsin-EDTA solution 0.05% (at 37 °C for 5 min) and removed by adding 300 μL of sterile LHC-9 medium. The cells were collected into a sterile tube and 800 μL of freezing solution (45% FBS, 4% DMSO, 2.5% Penicillin-Streptomycin, 1.5% HEPES 1 M in LHC-9) added before storing the cells at −20 °C.

Modified comet assay, with the use of endonuclease enzymes, i.e. endonuclease III (ENDOIII), formamidopyrimidine [fapy]-DNA glycosylase (FPG), and T4 endonuclease V (T4PDG), was performed according to the protocol previously described (Nozza et al., 2020). For each

Table 1
Exposure doses to PM1 and ultrafine particles during the RHAPS campaigns. Total number (#/cm^2) total mass (μg/cm^2) and total surface area (μm^2/cm^2) of particles deposited per surface unit (square cm) of the cultured cells were calculated (n = 240 from 6 min SMPS data) for each exposure day and considering total PM1 and UFP (diameter <100 nm) only. The columns UFP/PM1 reports the relative contribution of UFP to the PM1 for each exposure metric (number, mass, or surface area).

Date	Campaign	Exposure	Number (#/cm^2)			Mass (μg/cm^2)			Surface area (μm^2/cm^2)		
			PM_1	UFP (<100 nm)	UFP/PM_1	PM_1	UFP (<100 nm)	UFP/PM_1	PM_1	UFP (<100 nm)	UFP/PM_1
26 Jan	W1	RH1	14712990	14561249	0,990	0,0033	0,0008	0,2288	72,47	43,48	0,60
27 Jan	W1	RH2	13813361	13341096	0,966	0,0090	0,0014	0,1541	152,63	61,36	0,40
28 Jan	W1	RH3	15273543	14694069	0,962	0,0120	0,0015	0,1264	190,56	68,48	0,36
29 Jan	W1	RH4	9745946	8931678	0,916	0,0216	0,0012	0,0546	270,15	48,36	0,18
2 Feb	W2	RH5	11052590	10490436	0,949	0,0225	0,0011	0,0482	230,86	46,86	0,20
3 Feb	W2	RH6	5633033	5234850	0,929	0,0299	0,0006	0,0204	225,94	25,15	0,11
4 Feb	W2	RH7	10680666	10191871	0,954	0,0172	0,0008	0,0486	185,19	37,90	0,20
5 Feb	W2	RH8	8842124	8417935	0,952	0,0281	0,0007	0,0252	224,64	31,94	0,14
16 Feb	W3	RH9	11259587	10788479	0,958	0,0107	0,0010	0,0972	157,69	48,28	0,31
17 Feb	W3	RH10	9314540	8533563	0,916	0,0273	0,0010	0,0380	295,68	43,32	0,15
18 Feb	W3	RH11	9541767	8774992	0,920	0,0345	0,0011	0,0305	330,47	44,88	0,14
19 Feb	W3	RH12	8142356	7504430	0,922	0,0276	0,0010	0,0344	270,41	39,61	0,15
29 Jun	S1	RH13	5645683	5582343	0,989	0,0020	0,0005	0,2349	38,81	24,30	0,63
30 Jun	S1	RH14	10432955	10280539	0,985	0,0034	0,0008	0,2347	69,50	40,23	0,58
1 Jul	S1	RH15	10484605	10236500	0,976	0,0045	0,0010	0,2254	90,91	47,36	0,52
2 Jul	S1	RH16	6527222	6276158	0,962	0,0044	0,0008	0,1761	82,69	35,97	0,44

sample, 100 cells were analysed to quantify the tail moment with TriTek CometScore Freeware (Sumerduck, USA).

2.4. mRNA expression

Detailed description of the procedure followed to extract the mRNA from exposure and control samples are reported in (Aufderheide et al., 2017). Briefly, two inserts, one control and one exposed, were selected and cells were lysed with 300 μL of TriFast (EuroClone, Pero, Italy). Lysates were collected in sterile tubes and directly stored at −80 °C. The RNA was extracted via Direct-zol™ RNA MiniPrep Kit following the manufacturers' instructions (Zymo Research, Irvine, California). RNA amount and purity were evaluated by spectrophotometer (NanoVue Plus, Biochrom™, Cambridge, UK) calculating the 260/230 and 260/280 absorbance ratios. 300 ng of total RNA were retro-transcribed with random primers (Promega, Milano, Italy) and M-MLV Reverse Transcriptase Kit (Promega, Milano, Italy) into total cDNA, according to manufactures' indications. Analysis of the genes expression was carried out with 2 μL of cDNA using Luna® Universal qPCR Master Mix (New England BioLabs, Ipswich, USA), according to the primers listed in Table 1, and analysed on a CFX Connect Real-Time PCR Detection System (BIO-RAD, Hercules, USA). All reactions were run in triplicate and the relative abundance of the specific mRNA levels was calculated by normalising to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The complete list of genes and primer sequences is reported in Supplementary Table 2. All the sequences, which were obtained using Primer designing tool - NCBI e NIH and purchased by Metabion (Planegg, Germany). This restricted number of genes was selected considering the relevant biological pathways activated in *in vitro* and *in vivo* models exposed to PM.

2.5. Interleukin release

The release of IL-8 was analysed in the basal media of each exposed insert. At the end of the daily exposure medium from each well was collected and transferred to a sterile tube and immediately frozen at −20 °C. Human IL-8 ELISA Kit (ImmunoTools, Friesoythe, Germany) was used to quantify in duplicate the pg/mL of IL-8 released in the medium beneath the cells for each treated and control samples after 24 h of exposure to air pollutants.

2.7. Statistical analysis

Data are expressed as mean of medians ± SEM for the modified comet assay (n = 100 cells from each different technical replicate), and as mean ± SEM for the other experiments (n ≥ 3 from least three technical replicates). Statistical analysis was performed using GraphPad Prism 8.0.2 Software (GraphPad Software, San Diego, USA). Statistical differences were calculated using *t*-test considering the respective control for each exposed sample. Boxplot, correlation analyses and related statistical analyses (ANOVA with Tukey post-hoc) were performed with R (R Development Core Team, 2010). Finally, the R statistical environment was run to obtain dose-response curves for selected genes and relevant deposited doses parameters.

3. Results

3.1. Doses of exposure

Table 1 summarizes the exposure doses of PM_1 during each experiment, presented in terms of mass, number, and surface area, along with the relative contribution of ultrafine particles ($PM_{0.1}$).

During the winter campaigns, the average number of deposited PM_1 particles is $1.06 \pm 0.28 \times 10^7$ number/cm^2 significantly higher compared to the summer campaigns, where the average was $8.27 \pm 2.21 \times 10^6$ number particles/cm^2. Similarly, for ultrafine particles, the average deposited number of particles was slightly higher in winter ($1.01 \pm 0.29 \times 10^7$ number particles/cm^2) compared to summer ($8.09 \pm 2.18 \times 10^6$ particles/cm^2). Notably, the average PM_1 mass dose was significantly higher in winter ($2.03 \pm 0.99 \times 10^{-2}$ μg/cm^2) than in summer ($3.30 \pm 0.80 \times 10^{-3}$ μg/cm^2). Significant differences in the average deposited surface area were also observed between winter and summer campaigns (Table 1 and Supplementary Fig. 1). Specifically, the second and third winter campaigns (W2 and W3) were characterized, on average, by the highest deposited mass, while the first winter campaign (W1) showed the highest number of deposited particles. In contrast summer exposure (S1) recorded the lowest deposited mass but a median deposited number of particles comparable to W2 and W3.

3.2. Biological responses

Considering the four different campaigns of exposure as homogenous groups (W1, W2, W3, and S1 (Supplementary Fig. 1, average chemical properties), we observed that the release of IL-8 and the expression Cxcl-8 were significantly lower during summer exposures compared to winter exposures (Supplementary Fig. 2).

On the contrary, the daily expression of the selected genes showed significant differences (fold increase higher than 2 or lower than 0.5)

between treated and control cells during both winter and summer exposures (Fig. 1). Notably, all the genes analysed showed a different modulation across the different days of exposure. Oxidative stress-related genes (*HMOX* and *NQO1*) showed greater upregulation mostly during winter exposures. The Cxcl-8 gene, representative of the cell inflammatory responses, showed minor increases in winter but was rather downregulated in (compared to controls). DNA damage-related genes *ATM* and *Gadd45α* were upregulated in both seasons, with *ATM* showing the highest upregulation during winter exposure (February 4th relative fold increase equal to 3.8) and *Gadd45α* reaching the highest upregulation during summer (July 2nd relative fold increase equal to 6.9). IL-8 levels in the medium of exposed cells were modulated in winter (Fig. 1 and Supplementary Figs. 2 and 3), while summer PM_1 exposures determined an overall reduction of IL-8. Finally, DNA damage, assessed by comet assay, showed, in general, a greater impact of summer exposure compared to winter, though with some differences (Fig. 1 and supplementary Figure 2, 3 and 4). Specifically, the Comet_FPG had a different pattern than the other endpoints, showing increased damage during late winter exposures, in February, while the other assays showed higher effects in summer although increases were observed also during late winter experiments (for Comet_T4PDG and Comet_ENDO_III).

3.3. Associations between gene expression and dose of exposure variables

We first considered the associations between the doses of exposure to PM_1 or to $PM_{0.1}$ (commonly referred to as ultrafine particles of UFP) (Fig. 2A). Exposure doses are reported in terms of mass, number, or surface areas of deposited PM_1 (m_PM1, N_PM1, sa_PM1) and the mass, number, or surface area of deposited UFP (m_UFP, N_UFP and sa_UFP). The correlation coefficient (R^2) between exposure variable and genes expression showed a similar pattern, with positive association with the dose expressed as total N_PM1. The dose in term of deposited mass, m_PM1, also exhibited a positive correlation with the inflammatory markers *Cxcl-8* and IL8. A similar trend was observed considering the surface area as the exposure metric (sa_PM1 and sa_UFP). Specifically, oxidative gene *HMOX* showed a significant ($p < 0.05$) positive correlation with the deposited N_UFP and N_PM1 (R^2 equal to 0.27, with p-value of 0.04 and 0.037 for PM and UFP respectively). The release of IL-8 correlated positive with all exposure dose variables, showing significant correlation with the deposited PM_1 mass (R^2 equal to 0.27, p-value 0.039) and partial correlation with sa_PM_1 (R^2 equal to 0.22 and p-value $= 0.069$).

We then compared the dose-response (D-R) curves with the respective concentration-response (C–R) curves (Fig. 3 and Supplementary Fig. 5). Noteworthy, basing on mass and surface area metrics, the D-R and C–R curves are comparable, exhibiting similar trends in the

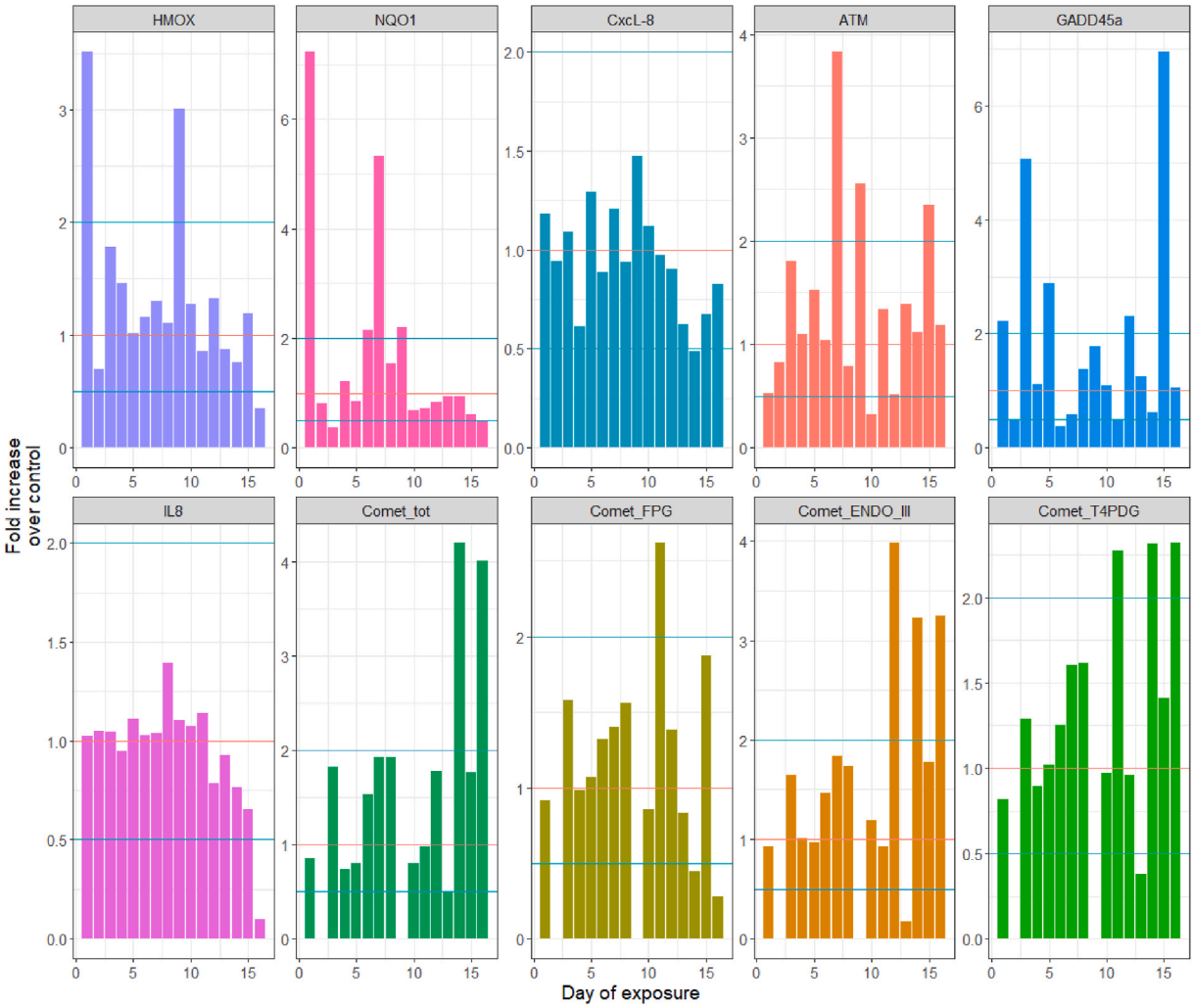

Fig. 1. Daily variation ($n \geq 3$) in gene expression and DNA damage outcomes during the different exposure experiments. The normalised control level (red straight line) and the reference values 2 folds and 0.5 folds variation (light blue straight line) are reported. Days 1–12 are the winter exposures while days from 13 to 16 correspond to summer exposures. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

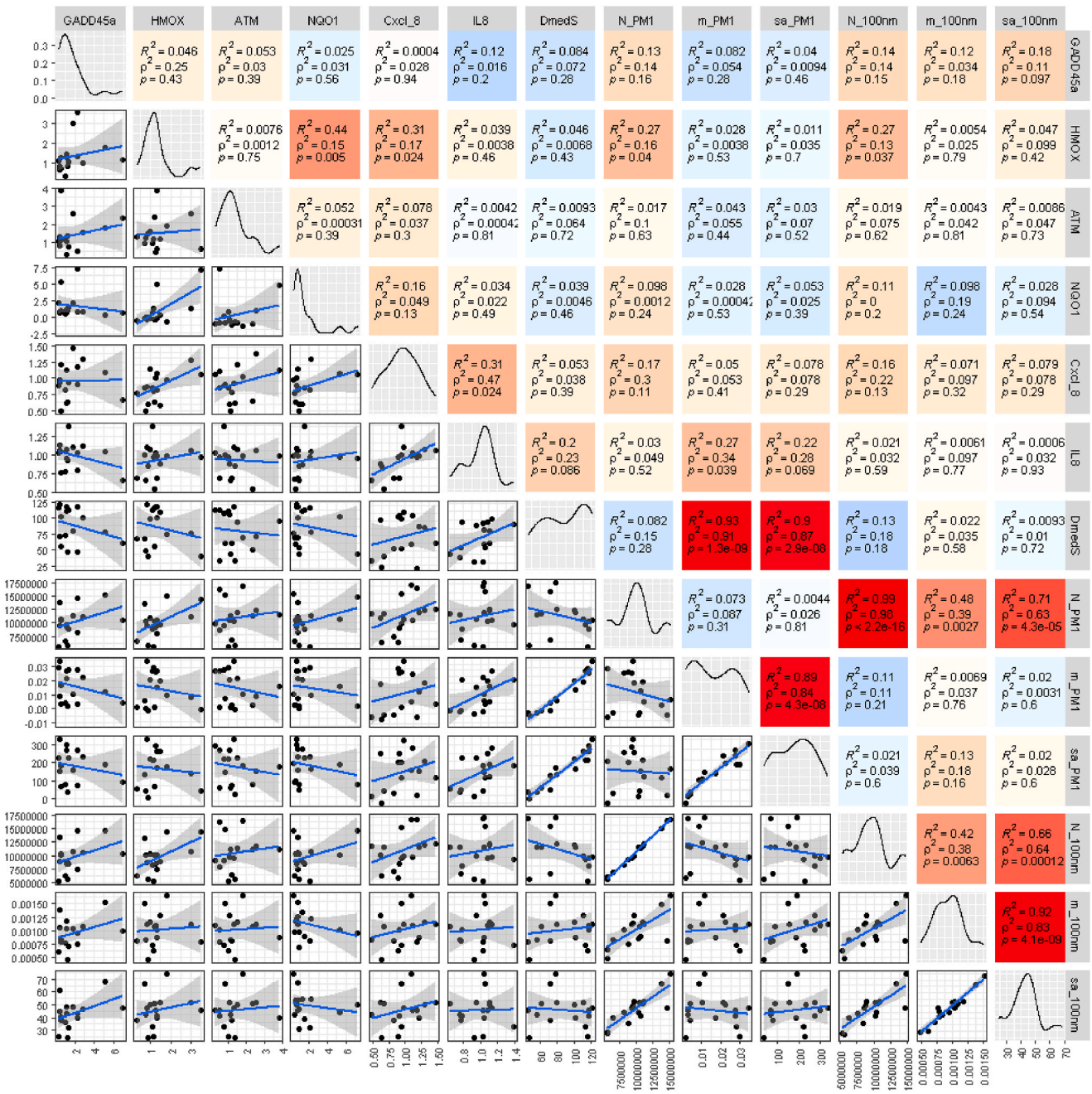

Fig. 2. Pearson (R2) and Spearman (ρ2) correlation matrix of modulated genes (A) and DNA damage endpoints (B) and exposure doses expressed as deposited number, deposited mass, or deposited surface area of the total PM1 and UFP particles (n = 16). Reddish background stands for a positive association while bluish background for negative ones. The significance of the association is reported as p value in each box. The diagonal plots report the data interpolated distribution of the data.

interpolating curves, for the number of particles the curves may differ substantially. This is particularly evident for the oxidative and inflammatory genes, *HMOX, NQO1* and *Cxcl-8*, and to some extent for the DNA damage-related gene *ATM* (Supplementary Fig. 5D). For these biological endpoints the D-R curves were almost linearly related to the exposure dose, whereas ambient concentrations (C–R curves) showed an inverted U-shape. RegardingIL8 protein release, the PM_1 mass-based D-R and C–R curves showed different relationships. The D-R curve indicated a smooth but continuous increase in IL8 release whereas the C–R curve once again suggested an inverted U-shaped relationship (Fig. 3).

Beside exposure doses, overall airborne PM_1 main chemical properties (mass fraction of black carbon BC – f_BC, mass fraction of sulphates – f_SO4; of nitrate – f_NO2; of ammonia – f_NH4 and mass fraction of organic carbon – f_OA, as obtained from the data of the AMS measurements as reported in (Costabile et al., 2023)) were correlated to the biological outcomes and results are reported in Supplementary Fig. 6.

3.4. Association between comet assay and dose of exposure variables

We also screened for the correlations between the Comet assay results and the exposure parameters described in previous paragraphs. Non-significant correlations were observed among most Comet assay endpoints (Comet, Comet_ENDOIII and Comet_T4PDG) and the PM_1 and UFP dose of exposure. Interestingly, Comet with FPG showed a unique

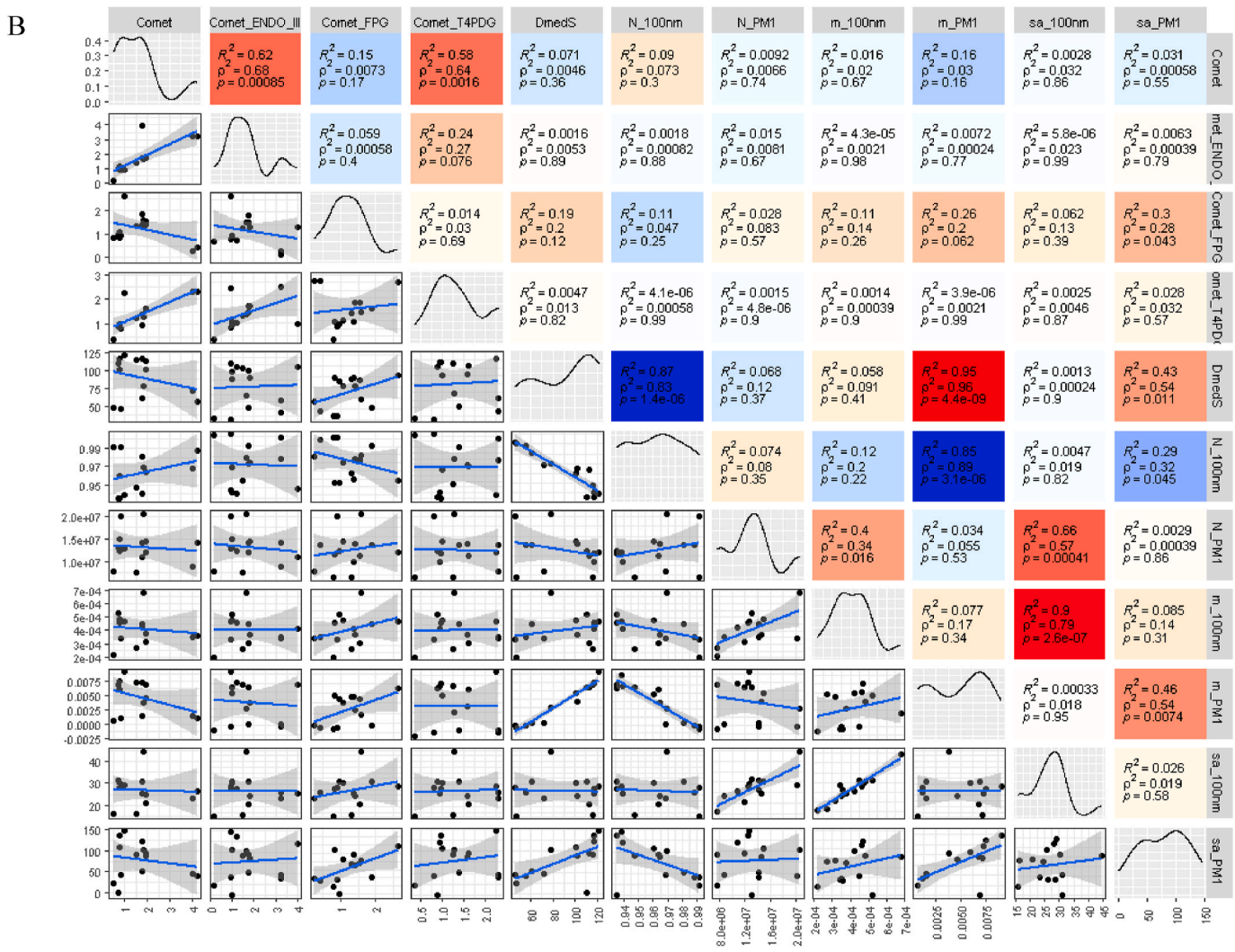

Fig. 2. (*continued*).

correlation pattern with positive, significant association with the deposited PM_1 surface area ($R^2 = 0.3$, $p = 0.043$) and, although not significant with the deposited PM_1 mass ($R^2 = 0.26$, $p = 0.062$) (Fig. 2B). Other deposition metrics did not show statistically significant results, although particles bigger than 100 nm had higher correlations than UFPs. Further, we explored the association between the DNA damage outcomes and the key chemical parameters characterized for ambient aerosol (Supplementary Fig. 7). According to the biological process behind DNA damaging effects of air pollution, significant positive correlations were reported between Comet ($R^2 = 0.56$ and $p = 0.0021$) Comet_ENDO_III ($R^2 = 0.26$ and $p = 0.061$) and Comet_T4PDG ($R^2 = 0.29$ and $p = 0.048$) and the chemical enrichment of PM_1 in organic aerosol (f_OA from AMS). Interestingly, among the different organic fractions, a significant positive correlation was reported between Comet ($R^2 = 0.52$ and $p = 0.0035$) Comet_ENDO_III ($R^2 = 0.29$ and $p = 0.049$) and Comet_T4PDG ($R^2 = 0.34$ and $p = 0.028$) and the chemical enrichment of oxidized organic aerosol species (f_OOA from AMS). Comet_FPG showed a specific pattern of correlation with positive association, although not statistically relevant, with enrichment of NO_3 (f_NO3, $R^2 = 0.27$ and $p = 0.056$).

3.5. Association between biological outcomes and size fractioned doses of exposure

Given the positive association of analysed genes with the number or the mass of UFP and PM_1, we further analysed correlations with additional size ranges (namely, 8–20 nm, 20–100 nm, 100–300 nm, 300–500 nm, and 500–1000 nm), representing a proxy parameter of the prevalence of the tiniest particles versus bigger ones (Supplementary Fig. 8). The correlations obtained using this new set of parameters are reported hereafter (see also Supplementary Fig. 8 for genes expression and IL8 release).

Among the different correlations, *HMOX* expression showed a significant positive association with the number, mass and surface area of UFP lower than 20 nm ($R^2 = 0.32$ and $p = 0.022$, $R^2 = 0.31$ and $p = 0.024$ and $R^2 = 0.32$ and $p = 0.023$, for number, mass and surface area respectively).

Positive correlations were also observed between the number of UFPs, between 20 and 100 nm, and *Gadd45α* expression ($R^2 = 0.24$ and $p = 0.054$), as well as UFPs lower than 20 nm and both *Cxcl-8* and *NQO1* expression ($R^2 = 0.19$ and $p = 0.093$ and $R^2 = 0.22$ and $p = 0.068$, respectively). Finally, the release of IL-8 was significantly associated with the number ($R^2 = 0.3$, $p = 0.028$) mass ($R^2 = 0.31$, $p = 0.024$) and surface area ($R^2 = 0.31$, $p = 0.025$) of particles between 500 and 1000 nm.

Analysing in detail the *HMOX* associations with finest UFPs and, considering the source-related associations reported in Costabile et al. (2023), it appears relevant the role of deposited primary fresh aerosol for both the number and the mass doses of deposited ultrafine particles (Fig. 4A and B). We found that higher *HMOX* gene expression occurs

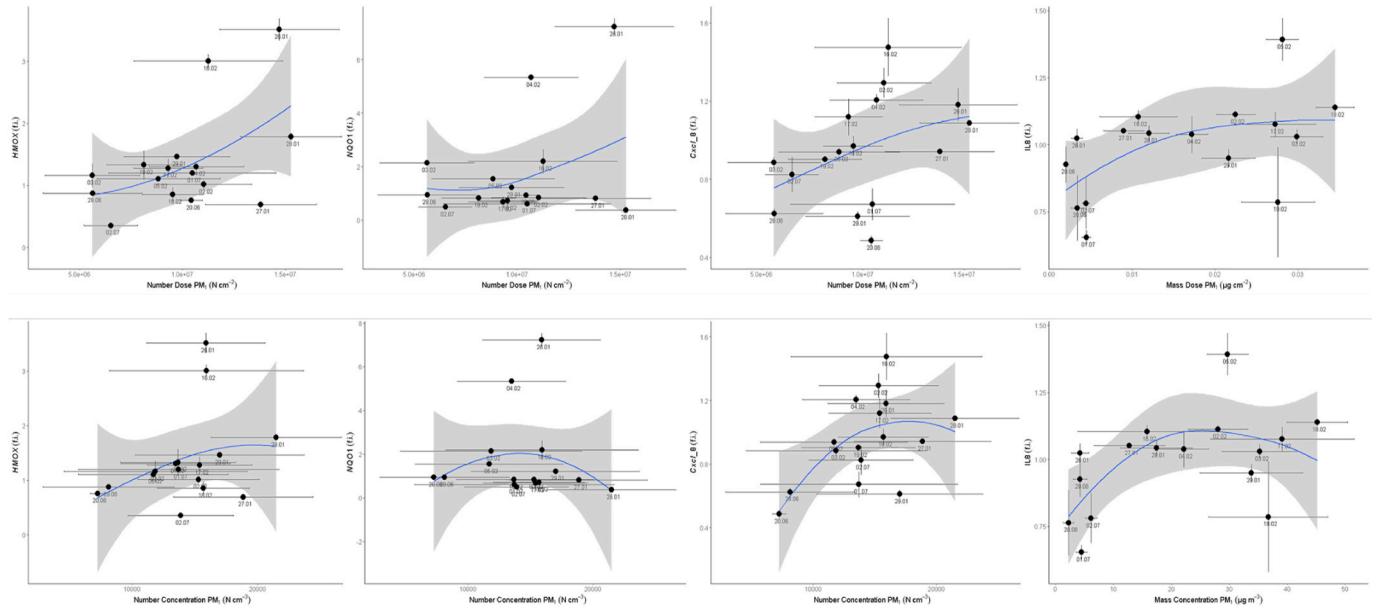

Fig. 3. Dose-Response (C–R, upper part of the figure) vs Concentration-Response (D-R, lower part of the figure) curves of selected genes and exposure variables, obtained from daily values (n = 16). The interpolation curves (in blue) with the relative confidence interval (grey area) are reported considering, for the different days of exposure, the ambient concentration (C–R curves) or the resulting dose of exposure (D-R curves). Only the response function curves showing differences are reported. Main differences are observed for the relation between ambient and deposited number of particles versus the expression of oxidative (HMOX and NQO1) inflammatory (Cxcl-8) genes. Noteworthy, for the antioxidant response genes, while the C–R interpolation shows reverse U-shaped curves the D-R report almost linear monotonic relations. The release of IL-8 protein is reported considering the C–R or D-R functions in term of mass of PM_1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

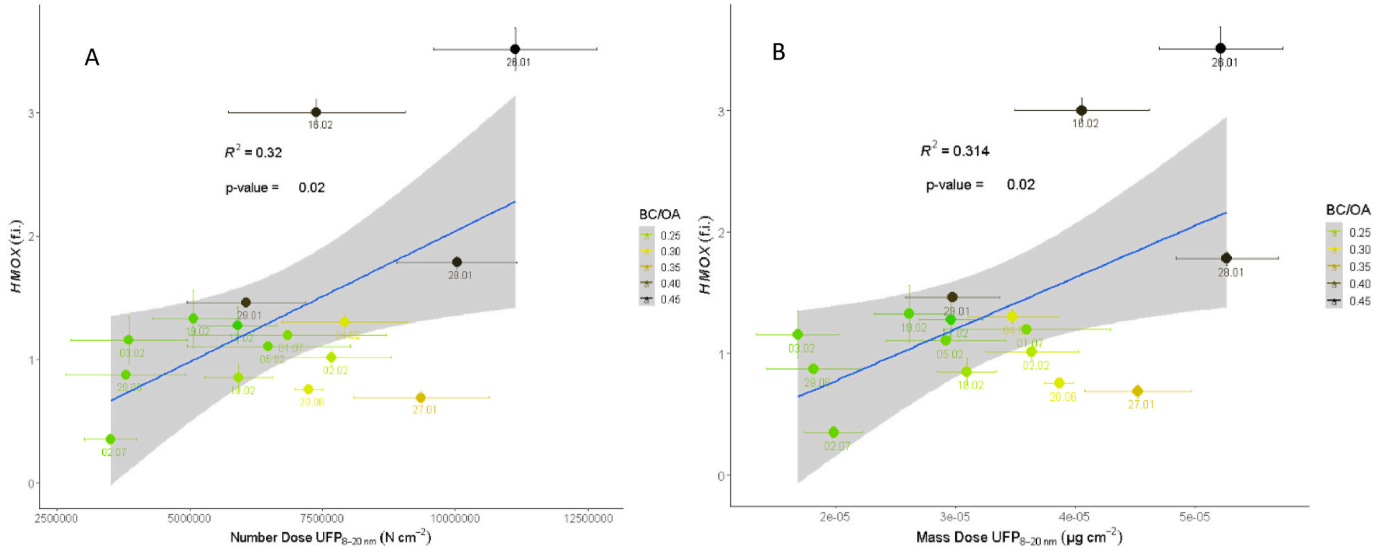

Fig. 4. Dose-response curves (linear fitting curve, n = 16) of HMOX vs number (A) or mass (B) of deposited UFPs between 8 and 20 nm. The curves, statistically significant underline the relevance of the tiniest particles of the UFP in driving the activation of the antioxidant gene. The effects are higher in the presence of freshly emitted particles (higher BC-to-OA, BC/OA, values) that have a higher pro-oxidant potential compared to the aged one (lower BC-to-OA, BC/OA, values).

with exposures characterized by a higher BC-to-OA ratio (representative of fresh urban aerosol), while a reduced expression is shown in presence of aged aerosol (low BC-to-OA) despite the nanoparticle exposure being the same (Fig. 4A and B). Moreover, the dose of exposure expressed as mass of deposited particles under 20 nm of diameter provided for a stronger association with *HMOX* gene expression than the mass of UFP or of PM_1 (Figs. 4B and 2A).

The release of IL-8 protein showed higher correlations with exposure to dose expressed as deposited mass ($R^2 = 0.31$, $p = 0.024$), surface area (R^2 0.31, $p = 0.025$) and number (R^2 0.30, $p = 0.028$) of particles between 500 and 1000 nm. The dose-response curve obtained considering the deposited mass of 500–1000 nm particles (Fig. 5), also reports the relative importance of the aged urban aerosol, possibly delivering a more complex chemistry. This specific size is, in fact, enriched during aging of fresh aerosol (Costabile et al., 2023) as also evidenced by the positive association of this biological endpoint with inorganic secondary ions (Supplementary Fig. 6).

Surprisingly, the Comet assay outcomes did not show specific association with the fractioned PM exposure doses (data not shown). However, Comet_FPG showed a distinct association with the different

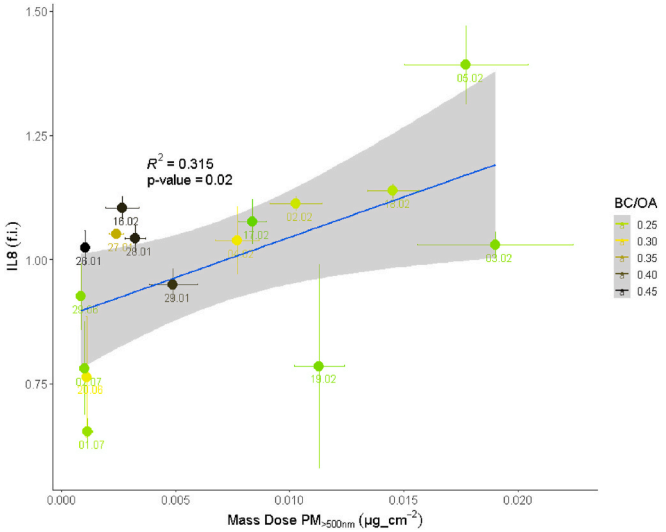

Fig. 5. Dose-response curve (linear fitting, n = 16) of IL8 vs the mass of deposited particles with diameter higher than 500 nm. The curve, statistically significant p = 0.02, although with a R^2 = 0.315, underline the relevance of the bigger particles in the size range we considered. The release of IL8, being mass dependent, may be primarily influenced by aerosol enriched in organic species also after aging processes (aging of particles and secondary aerosol formation as suggested by the higher IL8 release associated with low BC to OA ratio).

exposure doses although none of the association was strong and significant except, partly, for the association with the number of particles in the 500–1000 nm range (R^2 = 0.25 and p = 0.069).

All other results showed only a slight association with exposure doses, highlighting the importance of the complex PM chemistry in inducing DNA damage rather than specific PM size fractions. Examining the association between Comet assay results and PM_1 enrichment in organic aerosol (f_OA, Fig. 6), it is evident that the airborne PM concentration alone is not predictive of potential effects.

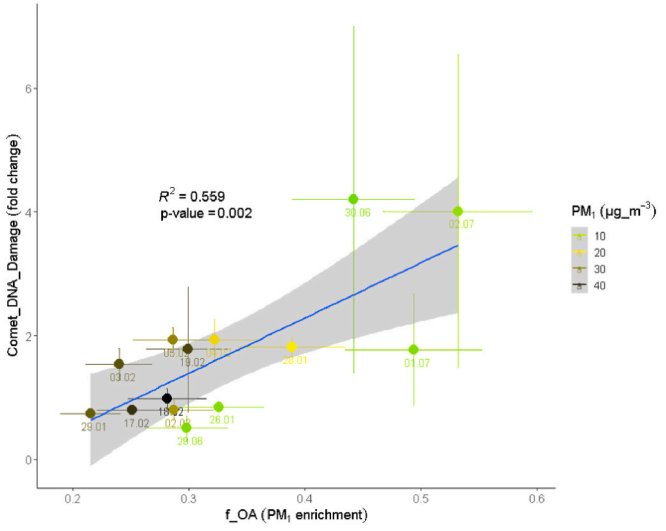

Fig. 6. Exposure-response curves of A) Comet assay (n = 14) results vs the enrichment of PM1 in organic aerosol (OA). The Comet-OA curve, statistically significant, and with a high R^2 = 0.559, underline the relevance of the chemistry determining atmospheric aerosol toxicological properties rather than the airborne mass, number, or surface area exposure doses. Noteworthy, higher DNA damaging effects were related to low PM_1 airborne concentrations.

4. Limitation of the study

We acknowledge some limitations of the present study. First, the *in vitro* model, although representative of a lung epithelial tissue is relatively simplicistic as it relies on a single cell type. More complex models, which take advantage of the interaction among various cell types, are becoming increasingly relevant. Advances in the field also include the use of organoids and lung-on-chip models (Xu et al., 2020; Rothen-Rutishauser et al., 2023; Yaqub et al., 2022; Silva et al., 2023; Doryab and Schmid, 2022; Petpiroon et al., 2023; Miller and Spence, 2017; Grytting et al., 2024). Additionally, the number of tests performed in each experiment could have been expanded, both in terms of parameters assessed, and number of replicates performed during each exposure. Given the fixed number of inserts per each exposure, we prioritized having a broader range of toxicological endpoints. As previously reported (Costabile et al., 2023) technical triplicates were always performed for each toxicological endpoint. Another limitation lies in the number of different exposures tested. Increasing the diversity of exposure could have strengthened the association observed in our study. Future experiments could enhance the robustness of our findings by adding additional data points to the curves presented here, thus improving the consistency of our work.

Furthermore, our study focused on only two seasons, with a greater number of experiments conducted in winter compared to summer. This imbalance may have influenced the results, potentially underestimating the effects of summer PM condition on the toxicological endpoints. Regarding limitations related to the particles characterization, the most relevant issues have been previously discussed in (Costabile et al., 2023) and we kindly refer readers to that publication.

5. Discussion

We present here a comprehensive evaluation of the dose-response association between realistic exposure doses in a lung epithelial *in vitro* model and selected biological outcomes that represent key biological processes. Obtaining data under human relevant exposure levels is pivotal for a proper risk assessment of defined compounds or pollutants (González Ortiz et al., 2021; Schulte et al., 2018; Paur et al., 2011). In this study, we compare different toxicological parameters, with ambient mass, surface area and particle, number concentration (ambient fine PM or UFP concentration) or with the dose (deposited particles at the *in vitro* model surface). Significantly, we show that the dose-response and concentration-response curves may vary depending on the metric and toxicological outcome considered. The more linear monotonic dose-response curves agree with the recent epidemiological evidence of a linear increase of the relative risk at low $PM_{2.5}$ exposure (Weichenthal et al., 2022), providing toxicological evidence of the adverse effect of $PM_{2.5}$ also at low doses of exposure. This is further supported by recent association curves reporting increased relative risks of hospital admission for relevant human disease to lagged fine PM exposure (Wei et al., 2024) and with the estimated association of global mortality burden associated to short-term exposure to fine PM worldwide (Yu et al., 2024). The burden of deaths in Europe is calculated as accounting on average for 1.53% of total regional death, with a higher burden in western (1.59%) than in other EU regions, while in northern America the burden is expected to be lower (1.07%) and in Africa and Asia it is expected higher (1.78% and 2.54%). This picture shows the urgency to better understand the mechanisms and the modes of action of ambient PM on health to tackle global short-term mortality as high as more than one million persons (Yu et al., 2024). Moreover, exposure-response curves are largely available in the literature, although mainly from epidemiology (Chen et al., 2023). On the contrary dose-response curves are scanty and the data we report here are of primary relevance for future risk analysis. Noteworthy, recent epidemiological results report a significant positive association between premature mortality risk and exposure to airborne nanoparticle concentration (Lloyd et al., 2024) and

with oxidative stress biomarkers during pregnancy in women exposed to fine PM with high oxidative potential (Meng et al.).

Despite $PM_{2.5}$ being recognized as a human carcinogen (Schmeisser et al., 2023) and increasing epidemiological evidence of its adverse effects at low mass concentrations (Weichenthal et al., 2022), current air quality standards and guidelines remain primarily focused on the mass of the airborne particles, without considering other possible metrics. Noteworthy, recent studies pinpoint the importance of analysing particle number concentration as drivers for specific adverse effects (Vallabani et al., 2023a; Lin et al., 2022; Zhang et al., 2022; Chen et al., 2016) or to offer better protection to sensible subpopulation (Pradhan et al., 2023). For instance, Vallabani and coauthors (Vallabani et al., 2023a) provided a first attempt to link the adverse health effects to UFPs number concentration and specific transport modes, showing the importance of car emissions also in association with co-exposure with other airborne compounds, such as viruses, bacteria, or pollens.

However, all these studies consider UFPs as a unique homogenous group. In contrast our study provides relevant evidence on the importance of sub-setting the UFPs into at least two groups i.e., particles smaller than 20 nm and those between 20 and 100 nm. Although previous research (Costabile et al., 2023) suggested the potential importance of an additional size range (from 20 to 40 nm), our analysisdid not reveal significantly different correlation compared to the two classes already defined (data not shown). In agreement to our results, a recent study (Wang et al., 2021) emphasized the importance of UFPs between 20 and 100 nm for pedestrian exposure in an urban environment. Moreover, the doses of exposure we reported align with the doses reported and expected by lung deposition models (Hofmann, 2011; Hama et al., 2017; Lepistö et al., 2023; Williams et al., 2011; Vu et al., 2017) making our findings relevant for hazard and risk assessment. In the context of next-generation risk assessment (Li et al., 2021a; Bajard et al., 2023; Sørli et al., 2022) the data we provide address at least three key points: i) to provide toxicological data using *in vitro* models; ii) to implement NAM that are relevant for the human exposure hazard and risk characterization and iii) to provide information at relevant human exposure doses.

The toxicological effects of UFPs (Schraufnagel, 2020; Kwon et al., 2020; Stone et al., 2017; Han et al., 2023; Araujo et al., 2008; Vallabani et al., 2023b; Juarez Facio et al., 2022a) are well documented, with numerous studies linking activation of inflammatory or pro-oxidant pathways in the lung or other organs. Our data strongly support the importance of considering selected size ranges of UPFs as an indicator of distinct aerosol types and sources to activate the antioxidant responsive element (ARE) pathway even after 24 h of exposure. Specifically, we observed that both the upregulation of *HMOX* and *NQO1* genes correlated with the number of deposited particles in the range 8–20 nm and 20–100 nm respectively, with stronger values for urban source related nanoparticles (i.e., traffic). The expression of these two genes is indeed highly correlated (Fig. 2), as expected for genes activated under the ARE pathway (Manzano-Covarrubias et al., 2023).

The associations with size fractioned UFPs were stronger than that obtained for the whole UFP range, supporting the relevance of considering specific ranges of ultrafine particles together with the relevant source apportionment to establish dose-response association. In previous work we used the condensation sink (CS), in association with other parameters such as the BC-to-OA ratio, to assess how certain toxic compounds, including ROS, condense onto larger or smaller particles (Costabile et al., 2023). Being the CS dominated by the accumulation mode particles (i.e. particles mostly contributing the mass concentration of PM1), a higher CS, correspond to a higher the PM_1 mass concentration, and vice versa. Therefore, a low CS, potentially coupled with a high BC-to-OA ratio, could indicate the enrichment of toxic compounds upon fresh nanoparticles (at low PM_1). Noteworthy, higher deposition of traffic-related nanoparticles (smaller than 20 nm) may occur at low mass ambient concentration of PM_1.

Consistent with this, it has been reported that the organic fraction associated with quasi ultrafine particles induce higher *HMOX* gene expression compared to $PM_{2.5}$ particles (Badran et al., 2020). Recent studies (Gualtieri et al., 2022; Marcella et al., 2022) further support the role of ultrafine particle below 20 nm in inducing oxidative species already after 20 min of exposure; similarly, others (Gualtieri et al., 2022; Hawley et al., 2014; Juarez Facio et al., 2022b) reported that the activation of the *HMOX* gene may be higher shortly after exposure to combustion derived particles. The rapid activation of this gene (from tens of minutes to few hours) compared to the time point of our analyses (24 h post-exposure) might explain the relatively low R^2 values in our dose-response curves. This suggests that reduced time of exposure during specific peaks of ultrafine particles concentration should be considered in the future.

The activation of inflammatory pathways after exposure to airborne pollutants has been largely reported (Li et al., 2021b; Glencross et al., 2020; Gangwar et al., 2020; Vogel et al., 2020) and the association between short-term exposure to high concentrations of larger particles (up to 700 nm) and increased hospital admission for respiratory and cardiovascular diseases has recently been proposed (Bergmann et al., 2023). Accordingly, we report that while inflammatory gene expression is associated to high particle numbers, the expression of the inflammatory proteins may be more specifically determined by the mass of particles reaching the lung epithelia. Notably, the activation dose differs between aged (higher mass dose necessary) and fresh (lower mass dose necessary) particles. Juarez-Facio and coauthors (Juarez Facio et al., 2022b), recently reported down regulation of IL-6 and IL-8 genes after 3 h post exposure in BEAS-2B cells, suggesting that different organic compounds may interact in determining the up or down-regulation of inflammatory genes. A similar down regulation was observed in a specific population exposed to volatile compounds and fine PM (Audi et al., 2017). We add here another piece of evidence that the number of UFPs or surface area of 8–20 nm particles may be better predictors of the inflammatory response activation, in term of gene expression upregulation, when the relative sources are properly apportioned. Being the particle size the same, biological responses are different for nanoparticles from traffic-related emission processing and nanoparticles from secondary processes, such as regional new particle formation events (Aufderheide et al., 2017). Nonetheless, given the complex interactions between cellular pathways, aged aerosol may support the release of inflammatory mediators at the protein level according to the mass deposition at the target lung tissue. In fact, while IL-8 coherently correlates to *Cxcl-8* gene expression (Fig. 2), the *Cxcl-8* gene is also associated with the *HMOX* gene expression, supporting a possible influence of the ARE in modulating the inflammatory gene response.

Airborne particles are known to induce DNA damaging effects (Xue et al., 2022). Here we report a contrasting effect of direct exposure to total DNA damage (Comet results) and oxidative DNA damage (Comet with FPG). A recent study (Bonetta et al., 2019) analysing the mutagenicity and genotoxic effects of the organic extract from quasi ultrafine PM showed the absence of increased oxidative damage running the Comet assay with FPG. The strong correlation between total DNA damage and the enrichment of PM1 in organic aerosol at low PM_1 mass concentration, points to the importance of primary organic components in determining the observed effects. The link between organic components of fine PM and cancer onset has been recently reviewed (Holme et al., 2023), underlying the complexity of the biological pathways activated upon polycyclic aromatic hydrocarbons exposure. The difference between Comet and Comet with FPG may be related to differences in the specific composition of the aerosol during the different days of exposure as partially reported by Velali et al. (2016). The higher effect of samples with a lower contribution from organic aerosol, but enriched with nitrate ions, in inducing oxidative DNA damage, may be partly explained by the pro-oxidant and pro-inflammatory effect of urban fresh emissions rather than secondary inorganic components, even though additional and more focused research is needed to sort-out this aspect. Finally, it has been suggested that epidemiological studies should go

beyond classical associations with airborne PM mass concentration considering, at least, also the composition of the particles (Weichenthal et al., 2024)

In conclusion, we present evidence of distinct associations between dose or concentration of exposure and toxicological outcomes. The relevance of developing dose-response curves is crucial for human protection and is highly needed for improving the health of exposed populations. Our findings are a primary attempt to contribute to the risk assessment of ambient airborne $PM_{2.5}$ by providing *in vitro* dose response curves generated using a novel approach, withing the broader framework of NAM, under conditions relevant for human exposure, including low exposure doses. This kind of application may be integrated into next-generation risk assessment approaches to improve our understanding about the impacts of air pollution, even at low concentrations, on exposed populations.

Finally, the data reported strongly support the need for novel metrics that go beyond the classical PM definitions (PM_1 or $PM_{2.5}$ or PM_{10}) or even the UFP ($PM_{0.1}$) one. For better protection and understanding of the impacts on human health novel metrics should be promoted and adopted in the future both at the research and legislation level, integrating for example the size distribution of particles in the atmosphere, considering not only the number concentration of UFPs but also the relative contribution of the tiniest particles. Moreover, information on the aerosol sources and on the atmospheric processing (aged vs fresh emission) possibly using the proxy parameter BC-to-OA. should be considered.

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CRediT authorship contribution statement

M. Gualtieri: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **G. Melzi:** Writing – original draft, Investigation, Formal analysis, Data curation. **F. Costabile:** Writing – original draft, Visualization, Formal analysis, Conceptualization. **M. Stracquadanio:** Writing – review & editing, Investigation, Data curation. **T. La Torretta:** Writing – review & editing, Investigation, Data curation. **G. Di Iulio:** Writing – review & editing, Visualization, Data curation. **E. Petralia:** Writing – review & editing, Investigation, Data curation. **M. Rinaldi:** Writing – review & editing, Investigation, Data curation. **M. Paglione:** Writing – review & editing, Investigation, Data curation. **S. Decesari:** Writing – review & editing, Project administration, Funding acquisition, Formal analysis. **P. Mantecca:** Writing – review & editing, Resources, Formal analysis. **E. Corsini:** Writing – review & editing, Supervision, Methodology, Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maurizio Gualtieri reports financial support was provided by Italian Ministry of the University. Maurizio Gualtieri reports financial support was provided by European Union NextGenerationEU, National Recovery and Resilience Plan (NRRP) Mission 4. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143417.

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