

## Redox-activity and *in vitro* effects of regional atmospheric aerosol pollution: Seasonal differences and correlation between oxidative potential and *in vitro* toxicity of PM<sub>1</sub>

Gloria Melzi<sup>a,\*</sup>, Lorenzo Massimi<sup>b,c</sup>, Maria Agostina Frezzini<sup>b,d</sup>, Martina Iulini<sup>a</sup>, Naima Tarallo<sup>a</sup>, Matteo Rinaldi<sup>e,f</sup>, Marco Paglione<sup>e,f</sup>, Emma Nozza<sup>a,g,h</sup>, Federica Crova<sup>i</sup>, Sara Valentini<sup>i</sup>, Gianluigi Valli<sup>i</sup>, Francesca Costabile<sup>f,j</sup>, Silvia Canepari<sup>b,c</sup>, Stefano Decesari<sup>e,f</sup>, Roberta Vecchi<sup>i</sup>, Marina Marinovich<sup>a</sup>, Emanuela Corsini<sup>a</sup>

<sup>a</sup> Department of Pharmacological and Biomolecular Science (DiSFeB) "Rodolfo Paoletti", Università degli Studi di Milano, Via Balzaretti 9, 20133 Milan, Italy

<sup>b</sup> Department of Environmental Biology, Sapienza University of Rome, P.le Aldo Moro, 5, 00185 Rome, Italy

<sup>c</sup> Institute of Atmospheric Pollution Research, National Research Council, Via Salaria, Km 29,300, Monterotondo St., 00015 Rome, Italy

<sup>d</sup> ARPA Lazio, Regional Environmental Protection Agency, Via Boncompagni 101, 00187 Rome, Italy

<sup>e</sup> Institute of Atmospheric Sciences and Climate, National Research Council, 40129 Bologna, Italy

<sup>f</sup> National Biodiversity Future Center, NBFC, 90133 Palermo, Italy

<sup>g</sup> Experimental Medicine, Università degli Studi di Milano, Via L. Vanvitelli 32, 20129 Milan, Italy

<sup>h</sup> Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Via della Commenda 19, 20122 Milan, Italy

<sup>i</sup> Department of Physics, Università degli Studi di Milano & INFN-Milano, Via Celoria 16, 20133 Milan, Italy

<sup>j</sup> Institute of Atmospheric Sciences and Climate, National Research Council, Via Fosso del Cavaliere 100, Rome, Italy

### ARTICLE INFO

Editor: Lawrence Lash

#### Keywords:

Particulate matter  
Oxidative potential  
Toxicology  
Inflammation  
Oxidative stress  
In Vitro

### ABSTRACT

Particulate Matter (PM) is a complex and heterogeneous mixture of atmospheric particles recognized as a threat to human health. Oxidative Potential (OP) measurement is a promising and integrative method for estimating PM-induced health impacts since it is recognized as more closely associated with adverse health effects than ordinarily used PM mass concentrations. OP measurements could be introduced in the air quality monitoring, along with the parameters currently evaluated. PM deposition in the lungs induces oxidative stress, inflammation, and DNA damage.

The study aimed to compare the OP measurements with toxicological effects on BEAS-2B and THP-1 cells of winter and summer PM<sub>1</sub> collected in the Po Valley (Italy) during 2021. PM<sub>1</sub> was extracted in deionized water by mechanical agitation and tested for OP and, in parallel, used to treat cells. Cytotoxicity, genotoxicity, oxidative stress, and inflammatory responses were assessed by MTT test, DCFH-DA assay, micronucleus,  $\gamma$ -H2AX, comet assay modified with endonucleases, ELISA, and Real-Time PCR. The evaluation of OP was performed by applying three different assays: dithiothreitol (OP<sup>DTT</sup>), ascorbic acid (OP<sup>AA</sup>), and 2',7'-dichlorofluorescein (OP<sup>DCFH</sup>), in addition, the reducing potential was also analysed (RP<sup>DPPH</sup>).

Seasonal differences were detected in all the parameters investigated. The amount of DNA damage detected with the Comet assay and ROS formation highlights the presence of oxidative damage both in winter and in summer samples, while DNA damage (micronucleus) and genes regulation were mainly detected in winter samples. A positive correlation with OP<sup>DCFH</sup> (Spearman's analysis,  $p < 0.05$ ) was detected for IL-8 secretion and  $\gamma$ -H2AX.

These results provide a biological support to the implementation in air quality monitoring of OP measurements as a useful proxy to estimate PM-induced cellular toxicological responses. In addition, these results provide new insights for the assessment of the ability of secondary aerosol in the background atmosphere to induce oxidative stress and health effects.

\* Corresponding author.

E-mail address: [gloria.melzi@unimi.it](mailto:gloria.melzi@unimi.it) (G. Melzi).

<https://doi.org/10.1016/j.taap.2024.116913>

Received 8 January 2024; Received in revised form 18 March 2024; Accepted 21 March 2024

Available online 24 March 2024

0041-008X/© 2024 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Ambient air pollution is recognized as a threat to human health, being responsible for >3 million premature deaths globally per year (with an increasing trend) (Lakey et al., 2016; Orru et al., 2017). Among atmospheric pollutants, particulate matter (PM) is the most complex for its heterogeneity in terms of size, chemical composition, origin, and chemical transformation in the atmosphere. In 2021, the World Health Organization (WHO) recommended reducing the level of PM<sub>2.5</sub> (i.e., particulate matter with an aerodynamic diameter lower than 2.5 µm) annual air quality guideline to 5 µg/m<sup>3</sup>. Indeed, long-term exposure even to low PM<sub>2.5</sub> concentrations showed positive associations with non-accidental, cardiovascular, non-malignant respiratory, and lung cancer mortality without an indication of a threshold (Stafoggia et al., 2022). In the same year, WHO prioritized studies on specific types of PM (i.e., black carbon, desert dust, and ultrafine particles) since recent studies demonstrated that defining threshold based on mass concentration resulted insufficient for defining new air quality guideline levels (World Health Organization, 2021). Air quality monitoring is required by international air quality regulations, as the European Directive 2008/50/EC or the United States Environmental Protection Agency Air Quality Index (EPA, 2024; European Parliament, 2008). PM mass concentration is still largely used as metrics to account for observed adverse biological and health effects, even though the toxicity of particles is more influenced by their chemical composition and dimension (Corsini et al., 2017; Marabini et al., 2017).

In recent year, oxidative potential (OP) of PM, defined as the ability of aerosol particles to oxidize target biomolecules, has been proposed as a more health relevant metric than PM mass concentration (Janssen et al., 2014; Yang et al., 2016). Different assays can be used to assess OP directly on particles, but there is still no agreement on the most representative assay to be used. Currently, the combined application of different acellular methods on the same samples is strongly suggested (Frezzini et al., 2019). The most commonly used acellular tests include the ascorbic acid (AA) test, the dithiothreitol (DTT) assay, and 2',7'-dichlorofluorescein (DCFH) assay (Campbell et al., 2019). The OP<sup>AA</sup> and OP<sup>DTT</sup> assays are based on the depletion, over time, of AA and DTT antioxidants added to PM samples, while the OP<sup>DCFH</sup> assesses the content of particle-bound ROS by determining the intensity of the DCF fluorescence signal over time due to the enzyme-catalyzed oxidation of DCFH (non-fluorescent) by ROS in PM samples. Several studies have provided a wide database of OP results to study different redox components of PM, showing the different sensitivity of the test to the pathways of reactive oxygen and nitrogen species (ROS/RNS) formation (Calas et al., 2019; Frezzini et al., 2019; Li et al., 2003). AA assay has been shown to be most sensitive to coarse particles rich in transition metals (e.g., Fe, Mn, Mo and especially Cu); DTT assay has been strictly associated with the presence of polycyclic aromatic hydrocarbons (PAHs), quinones, transition metals, and secondary organic aerosols (SOA), and DCFH assay has been shown to be most sensitive to fine particles rich in organic compounds such as PAHs, and also some metals or their oxides (Calas et al., 2018; Charrier and Anastasio, 2012; Janssen et al., 2014; Kramer et al., 2016; Perrone et al., 2016; Verma et al., 2015). While the knowledge about the relationships between OP and PM-induced toxicological effects are limited (Frezzini et al., 2019). It is, therefore, important to investigate the correlation between the acellular measurement of OP of PM and its effects on lung cells, such as oxidative stress, genotoxicity and inflammation. Moreover, the ability of particle-bound ROS and secondary aerosol in the background atmosphere to induce oxidative stress and health effects has been poorly evaluated to date and deserves further investigation.

At cellular level, the adverse effects of PM depend on its physico-chemical properties (Møller et al., 2014; Park et al., 2018). PM exposure have been associated with alteration of the cellular redox, generation of ROS, DNA oxidative damage, mutagenicity, stimulation of pro-inflammatory mediators, etc. (Corsini et al., 2019; Corsini et al., 2017;

Corsini et al., 2013; Costabile et al., 2023; Marabini et al., 2017; Møller et al., 2020). The generation of ROS and/or the inadequate antioxidant defences is studied both with the use of fluorescent probe (Jung et al., 2012; Longhin et al., 2013a) and with the analysis of mRNA expression of genes involved in the antioxidant response (i.e., nuclear factor erythroid 2-related factor 2 – NRF2 – heme oxygenase 1 – HMOX – and NAD(P)H quinone dehydrogenase 1 – NQO-1) (Abbas et al., 2019). Particles exposure leads to up-regulation of cytokines including interleukins 6 and 8 (IL-6, IL-8), and tumour necrosis factor-alpha (TNF-α), which have been detected after exposure to particles of both monocytes/macrophages and epithelial cells (Velali et al., 2016). DNA damage is induced following PM exposure via oxidation of bases or breaks at the single or double strand of the helix. The induction of DNA damage can have different outcomes: it can be rescued when genes involved in DNA repair (as Ataxia-Telangiectasia Mutated – ATM – and Growth Arrest And DNA Damage Inducible Alpha – GADD45α) are activated (Hartwig et al., 2020; Rosssner et al., 2015), or it can lead to more severe DNA damage induction with the consequent cellular death through apoptosis or necrosis, or with a mutagenic effect (Peixoto et al., 2017).

In this context, the Italian project RHAPS (Redox-activity and Health-effects of Atmospheric Primary and Secondary aerosol), launched in 2019, has as major objective the identification of specific PM<sub>1</sub> properties from anthropogenic sources, with particular interest on secondary organic aerosol (SOA), responsible for toxicological effects and that can be used as new metrics for health-related outdoor pollution studies. Secondary organic and inorganic compounds are major constituents of regional aerosol pollution, affecting the background concentrations of fine PM in geographical areas characterized by distributed anthropogenic sources and scarce atmospheric ventilation. In one of such areas, the Po Valley (Italy), measurements of ROS in PM<sub>1</sub> and fog water highlighted the potential role of SOA in triggering oxidative stress in lung cells (Decesari et al., 2017). In the frame of RHAPS, field campaigns and laboratory experiments were carried out with a focus on finding a link between the OP carried by water soluble particle components and PM<sub>1</sub> *in vitro* toxicity. The study focused on PM<sub>1</sub>, as because secondary organic and inorganic compounds enrich in this size fraction with lesser influence from crustal material, it is considered the main responsible for adverse health effects and a promising metric compared to PM<sub>10</sub> or PM<sub>2.5</sub>. Within the project, two field campaigns in two seasons were carried out with a focus on finding a link between OP and PM<sub>1</sub> toxicity (Costabile et al., 2022). Results obtained are the object of the current manuscript.

In Costabile et al. (2023) we analysed HMOX and CXCL-8 gene expression in BEAS-2B cells exposed in the Air-Liquid Interface (ALI), and found higher values for higher levels of PM<sub>1</sub> total OP enriched in nanoparticles. In this study, we provide a wider dataset of chemical and toxicological measurement that, as far as the author known, was never reported before. >150 samples were characterized both chemically and toxicologically.

## 2. Materials and methods

### 2.1. Cell culture

The human non-tumorigenic lung epithelial cell line derived from human lung tissue BEAS-2B cells (catalogue n. 95,102,433, Sigma Aldrich, Darmstadt, Germany) were grown on CELL-BIND® 75 cm<sup>2</sup> flasks (Corning, New York, USA) in LHC-9 medium (catalogue n. 12,680,013, Gibco™, Waltham, USA) supplemented with 1% of penicillin/streptomycin solution (catalogue n. ECB3001, EuroClone, Pero, Italy). The human monocytic THP-1 cell line was obtained from Istituto Zooprofilattico (Brescia, Italy). For experiments, THP-1 cells were diluted to 10<sup>6</sup> cells/mL in RPMI 1640 (catalogue n. R8758) containing 2 mM L-glutamine (catalogue n. 59202C1 of penicillin/streptomycin solution, 50 µM 2-mercaptoethanol (catalogue n. M3148), supplemented

with 10% heated-inactivated foetal calf serum (catalogue n. F7524). Cells were maintained at 37 °C in a 5% CO<sub>2</sub> atmosphere and sub-cultured once reaching 80% confluence for BEAS-2B and three times a week for THP-1.

## 2.2. PM<sub>1</sub> collection

PM collection is described in Costabile et al. (2022). Briefly, PM<sub>1</sub> was collected at two sites in the southern Po Valley, namely Bologna (44°31'29" N, 11°20'27" E) and San Pietro Capofiume (44°39'15" N, 11°37'29" E). These sites were chosen to represent a background urban location and a rural area, respectively, due to their strategic position in the Po Valley in Italy, where the pollutants often accumulate due to frequent poor atmospheric dispersion conditions making it one of the major air pollution hotspots in Europe (Vecchi et al., 2007). The winter campaign took place from the 21st of January to the 18th of March 2021, while the summer campaign from the 8th of June to the 14th of July 2021. During wintertime parallel sampling was performed at the two sites, while in summer only the background urban site of Bologna was investigated. The samples devoted to toxicological assays and to OP assessment were collected on Teflon filters (PTFE, Pall R2PJ047, Pall Life Sciences, Ann Arbor, USA) with tandem dual channel sequential samplers (model Gemini, Dadolab, Italy) with PM<sub>1</sub> inlet. In parallel, also samples on pre-fired 47 mm diameter quartz fibre filters (Pallflex Tisuquartz 2500 QAO-UP, Pall Life Sciences, Ann Arbor, USA) with Single channel (TCR-Tecora Skypost) were collected for some additional OP tests. All samples were characterized as for elements, ions, and elemental and organic carbon. Details on the monitoring campaigns are reported in detail in Costabile et al. (2022).

The recovery and storage of the filters was done once a week. During the sampling campaigns, not sampled filters were kept along with the sampled filters during all experimental phases as field blanks (or blank control, BC), and were used as a control for all the toxicological analysis. It is noteworthy that OP measurements and toxicological assays were performed exactly at the same time, i.e., in parallel at the two laboratories devoted to these tests, in order to avoid any bias due to differences in storage and extraction times.

## 2.3. PM<sub>1</sub> extraction

Each PTFE filter was extracted in 10 mL of deionized water by rotating agitation at 60 rpm for 30 min. The obtained solution was then filtered through a nitrocellulose filter (pore size 0.45 µm, Merck Millipore Ltd., Billerica, MA, USA) and split into proper aliquots to perform the different tests (Costabile et al., 2022).

For toxicological analysis, 1.5 mL of sterile deionized sterile water was used for the extraction in a Petri's dishes 60 mm. The filters were posed on the surface of water with the side where the particles were collected in contact with the water. Each Petri's dish was sealed with parafilm and posed on the mini-shaker (PSU-27 mini shaker – Biosan, Riga, Latvia) for mechanical agitation for 30 min at 300 rpm. At the end of the 30 min extraction, the filters were removed from the Petri's dishes and discharged, while the 1.5 mL water-soluble extracts were collected in a 2 mL sterile Eppendorfs and stored at –20 °C until use. During the campaigns, control filters were collected as stated in the section "PM<sub>1</sub> collection" and treated in the same manner as the PM<sub>1</sub> sampled ones.

Filter extraction for OP and toxicological investigation were conducted in parallel at the same time to avoid possible bias due to differences in storage times.

## 2.4. Treatments

BEAS-2B cells were seeded and allowed to grow for 48 h before treatments. PM-extracts were thawed before use and vortexed for 10 s to allow the resuspension of possible particles in the suspension. Both BEAS-2B and THP-1 cells were exposed to the extracted water-soluble

PM diluted 1:10 in LHC-9 or RPMI 1640 for 24 h, where not differently stated. The toxicological data shown in this manuscript are not normalized on the mass of PM sampled during the collection to highlight the possible daily differences on biological activity.

## 2.5. OP tests

Water-soluble OP (OP<sup>AA</sup>, OP<sup>DTT</sup>, OP<sup>DCFH</sup>) and RP (RP<sup>DPPH</sup> - 2,2-diphenyl-1-picrylhydrazyl) measurements were performed on PM<sub>1</sub> PTFE filters as described in Costabile et al. (2022), which followed analytical methods extensively detailed by Frezzini et al. (2022, 2019) and Massimi et al. (2020). In addition, a sampling line mounting quartz fibre filters was devoted to water-soluble OP determination by the DTT assay (OPQ<sup>DTT</sup>). The adopted procedures are those by Cho et al. (2005) and Verma et al. (2009). Obtained values were normalized by sampling volume.

## 2.6. Cytotoxicity

Cell viability was assessed using the MTT reduction test (Gerlier and Thomasset, 1986), following the method described in Melzi et al. (2023) and supplementary materials. Cells were seeded in 96-well plates and exposed to PM<sub>1</sub> (1:10 in cell culture medium) for 24 h.

## 2.7. Reactive oxygen species formation

To evaluate oxidative stress induction in treated cells, the ROS formation assay was performed and combined with Lowry method for protein quantification. The DCFH-Diacetate (DA) method was used. This method was used as a simple and cost-effective way to detect ROS in cells (Kim and Xue, 2020). 1 × 10<sup>4</sup> cells were seeded on two different 96-well black plates (Brand, Wertheim, Germany) and – after 72 h – treated for 30 min and 1 h with PM<sub>1</sub> (1:10 in cell culture medium). This protocol uses the 2',7'-Dichlorofluorescein diacetate (DCFH-DA, catalogue n. D6883, Sigma Aldrich, Darmstadt, Germany) as a fluorogenic probe in a concentration of 50 mM in DMSO (catalogue n. 4540, Sigma Aldrich, Darmstadt, Germany), then diluted 1:2000 in medium. After 30 min and 1 h, cells were washed with PBS and fluorescence was detected with the spectrofluorometer EnSpire (Perkin Elmer, Waltham, USA) at 495 nm wavelength. Fluorescence values were normalized on the protein content of each sample, obtained by Lowry method (Lowry et al., 1951). Results are expressed as FU/µg proteins (fluorescence units on micrograms of proteins).

## 2.8. Alkaline Comet assay modified with endonucleases

The Comet assay is a single cell gel electrophoresis method, which allows to identify DNA damage, like single and double strand breaks, cross-linking bounds, and apurinic/aprimidinic sites (Cordelli et al., 2021; Singh et al., 1988; Tice et al., 2000). The alkaline assay is performed with a basic buffer (pH > 13), to identify DNA damage at single and double helix, as well as alkali-labile sites. 1.5 × 10<sup>4</sup> cells were seeded in a 96-multiwell coated with a collagen solution and – after 72 h – treated for 24 h. Comet assay was performed as previously described, and reported more in detail in the supplementary materials (Nozza et al., 2020). Experiments were read with fluorescence microscopy (AxioVision), 40× oil magnification. 100 cells were analysed for each sample using TriTek CometScore™ software, and data are reported as mean of the median of the tail moment values.

## 2.9. Assessment of genotoxicity: evaluation of H2AX phosphorylation and micronuclei detection

The presence of double strand breaks and micronuclei was performed using immunofluorescence techniques. 5 × 10<sup>5</sup> cells per well were seeded in a 24-multiwell on pre-coated 12 mm round cover slide.

The protocol was previously described in Melzi et al. (2023), and reported in detail in the supplementary material. Slides were read with 100× oil magnification with fluorescence microscopy (Axiovert 200 M) using DAPI and FITC filters. To obtain data for the  $\gamma$ -H2AX analysis, it was analysed a total of 100 cells for each sample. The same samples were used for the evaluation of the presence of micronuclei, 1000 cells were counted for each sample, and analysed following the criteria described by Fenech (Fenech, 2000), to assess the spontaneous formation of micronuclei following PM-treatment.

### 2.10. Interleukin-8 (IL-8) release

The release of IL-8 was measured following 24 h of treatment. Supernatants were collected from the multi-well used for the immunofluorescence or for RNA extraction and stored at  $-20^{\circ}\text{C}$  until evaluation. The secretion of the pro-inflammatory cytokine IL-8 was assessed by a commercially available kit following supplier's instructions (ImmunoTools, Friesoythe, Germany). Tetramethylbenzidine solution (catalogue n. T4444, Sigma Aldrich, Darmstadt, Germany) were added to start the final colorimetric reaction. Plates were read at 595 nm in absorbance with the microplate reader (Molecular Devices, Emax precision microplate reader, San Jose, USA). Results are expressed in pg/mL IL-8 secretion. IL-8 secretion was tested also in THP-1 cells. Results obtained on this parameter are reported in Costabile et al. (2022) and used in this manuscript for correlation analysis.

### 2.11. Gene expression analysis

RNA extraction was performed using Direct-zol™ RNA MiniPrep (catalogue n. R2053, Zymo Research, Irvine, USA).  $1 \times 10^5$  cells were seeded in a 12-multiwell and let grow for 72 h. After 24 h of treatment, TRiFast II (catalogue n. EMR517100, EuroClone, Pero, Italy) was used to lyse the cells. RNA was extracted following the protocol provided by the kit. Quantification of RNA was performed with Nanodrop (GE Healthcare, Chicago, USA). mRNA extracted was retrotranscribed using Promega reagents and protocol: Random Primer (catalogue n. C118A, Promega, Madison, USA), dNTPs (Amersham™, dNTPs Polymerisation mix, catalogue n. GEH28406557, GE Healthcare, Chicago, USA), Buffer 5× (catalogue n. M531A, Promega, Madison, USA), DNase/RNase free water, and M-MLV Reverse Transcriptase RNase enzyme (catalogue n. M369B, Promega, Madison, USA). Real Time Polymerase Chain Reaction (RT-PCR) was performed. As housekeeping, *GAPDH* gene was used (Barber et al., 2005; Panina et al., 2018), and the Ct data of this gene for all the tested samples can be found in the supplementary materials. cDNA was mixed with Master mix – prepared using Luna Taq (catalogue n. M3003X, New England BioLabs, Ipswich, USA), DNase/RNase free water and Oligonucleotide Forward and Reverse (MetaBion GmbH, Planegg, Germany, reported in Supplemental Material, qRT-PCR primers Table S1) diluted 1:20 in DNase/RNase free water – at a concentration of 3 ng/ $\mu\text{L}$ . The solution was added in a 96-multiwell for PCR (EuroClone, Pero, Italy), which was centrifuged at 1000 rpm for 2 min before reading with CFX Real Time PCR Detection Systems (BioRad, Hercules, USA) using the software CFX Maestro v2.3 (BioRad, Hercules, USA). The PCR reading cycle was 15 s at  $95^{\circ}\text{C}$ , 30 s at  $60^{\circ}\text{C}$ , 10 s at  $65^{\circ}\text{C}$  repeated 50 times and in the end 5 s at  $95^{\circ}\text{C}$ .

### 2.12. Statistical analysis

All cellular experiments were repeated at least three times. Data are reported as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was performed using software GraphPad Prism v9.0.0 (GraphPad Software, San Diego, USA). One-Way ANOVA test was chosen for the analysis of all results, in association with Dunnett's Multiple Comparison post hoc test. For the comet assay Two-Way ANOVA, in association with Dunnett's multiple comparison post hoc test was used. Spearman's Correlation analysis was performed to correlate the toxicological data

with OP results. Results were considered significant at  $p < 0.05$ .

## 3. Results

### 3.1. Oxidative potential

OP results are shown in Fig. 1.  $\text{OP}^{\text{DCFH}}$  and  $\text{OP}^{\text{DTT}}$  values (Fig. 1B and D) are comparable between the two sampling sites. Furthermore, results of the two OP assays exhibit a seasonal pattern: values from the urban samples are generally higher in winter than in summer, likely due to the major contribution of biomass domestic heating, to which DCFH and DTT are particularly and notably sensitive (Calas et al., 2018; Campbell et al., 2021; Perrone et al., 2016) and to higher atmospheric stability during winter, which increases  $\text{PM}_{10}$  mass concentration. On the contrary,  $\text{OP}^{\text{AA}}$  values (Fig. 1C) are very low at both sites and indicatively equivalent between winter and summer in the urban area; in general, they were higher in the rural area than urban during winter. This is in agreement with the fact that, as already mentioned, in previous literature works the AA test results correlate mostly with the PM coarse fraction enriched in transition metals while here the focus is on the sub-micron sized particles, whose largest contributors are secondary aerosol components like nitrates, sulphates, and organic matter.

$\text{OPQ}^{\text{DTT}}$  time variability (Fig. 1E) is generally comparable between the two areas during winter, even though the median  $\text{OPQ}^{\text{DTT}}$  is slightly lower in rural area than urban. On the other hand, a decrease is observed in urban area from winter to summer, in analogy with the trends of  $\text{PM}_{10}$  mass concentration. Fig. 1F shows the results obtained with the analysis of  $\text{RP}^{\text{DPPH}}$ . The values related to this parameter is higher in winter than in summer.

It is worth highlighting that  $\text{OP}^{\text{DTT}}$  values are generally higher when DTT assay is performed on PTFE filters instead of quartz filters. More insights into OP assays response will be addressed and discussed in a further related paper.

### 3.2. Cell viability

BEAS-2B cells have been used as a surrogate of lung epithelial cells and exposed to water-extracts of  $\text{PM}_{10}$  diluted 1:10 in complete medium for 24 h. Cell viability was assessed using the MTT assay and results are shown in Fig. 2A. Results are expressed as % of blank control. Seasonal and site differences on cell viability were not statistically significant, even if some sample collected in the rural area were able to reduce the cell viability below the 70%. For the majority of samples, cell viability was similar to control values.

### 3.3. Oxidative stress

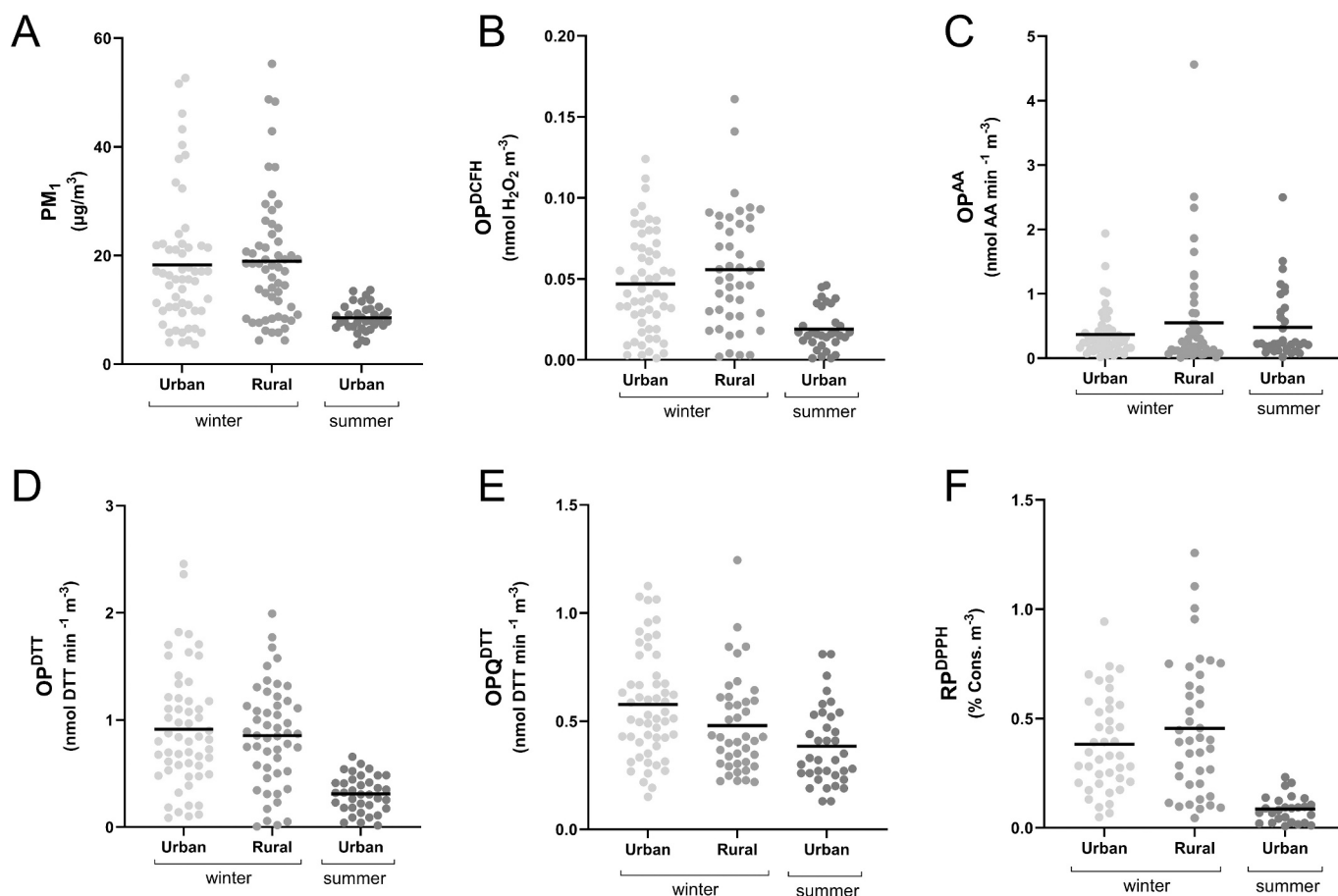
Fig. 2B shows the results of ROS formation following exposure to  $\text{PM}_{10}$  samples. ROS production was investigated at 30 min and 1 h. Most of the samples showed a slight increase of ROS formation (approximately 20%). For some samples, no ROS production was detected. Winter and summer samples showed similar trends with no statistical differences between sites.

The gene expression of HMOX and NQO-1, both induced in response to an oxidative stress, was evaluated after 24 h of treatment and results are shown in Fig. 2C and D, respectively. Increased expression of HMOX was observed in some samples in winter, while during the summer campaign, only few days showed increase of HMOX expression. NQO-1 was upregulated in winter and in summer in both sites, with a statistically significant difference between rural area winter campaign and urban area summer campaign.

### 3.4. Genotoxicity and DNA repair activation

Cells were treated for 24 h with the extract from  $\text{PM}_{10}$ . Fig. 3A shows the results of the immunofluorescence for  $\gamma$ -H2AX to detect double





**Fig. 1.** Seasonal and site differences of  $PM_{10}$  concentration and OP assays. (A)  $PM_{10}$  mass concentration expressed in  $\mu\text{g}/\text{m}^3$ . (B–F) Average responses of oxidative potential and reducing potential assays. Results are normalized on the volume samples on filter and reported in  $\text{nmol H}_2\text{O}_2/\text{m}^3$  (B,  $OP^{DCFH}$ ), in  $\text{nmol AA}/\text{min m}^3$  (C,  $OP^{AA}$ ), in  $\text{nmol DTT}/\text{min m}^3$  (D,  $OP^{DTT}$ ), in  $\text{nmol DTT}/\text{min m}^3$  (E,  $OPQ^{DTT}$ ), and in  $\% \text{ cons}/\text{m}^3$  (F,  $RP^{DPPH}$ ).

strands breaks, reported as percentage of nuclei with  $>10$  foci of  $\gamma$ -H2AX (representative images are reported in Fig. S1). Increase of DNA damage was detected mainly in the samples collected during the winter campaign. Considering all the results, during the summer campaign, the increase of DNA damage detected by H2AX phosphorylation was less than in winter, and these differences are statistically significant. Fig. 3C shows the micronuclei assay results. All the sample showed increase of this type of DNA damage compared to the blank controls, with no differences between winter and summer. Results of the gene expression analysis of GADD45 $\alpha$  and ATM are reported in Fig. 3B and D respectively. Upregulation and downregulation of these genes' expression are present in both the campaigns and sites with few dates in which a strong upregulation (fold-change  $>2$ ) is observed. While no statistically significant differences were detected in ATM expression among the different campaign, the increase of GADD45 $\alpha$  expression in the winter campaigns in both sites is statistically significant compared with the expression of the same gene in summer, which is in agreement with the DNA damage as assessed by the formation of  $\gamma$ -H2AX foci.

Fig. 4 shows the comprehensive results of the alkaline Comet assay modified with endonucleases. Treated samples show increase DNA damage compared to controls. Furthermore, the treatment with the enzymes ENDO III (urban – winter) and FPG (rural – winter, and urban – summer) highlight the presence of oxidative damage both in winter and in summer, and this is showed by the statistically significant increase of the level of DNA damage in the mean of the enzymatically treated samples compared to the mean of the not treated.

### 3.5. Inflammation and MUC5 AC and ACE-2 expression

CXCL-8 was upregulated only in few days during all the campaigns (Fig. 5A), while an increase secretion of IL-8 was detected which resulted statistically significant in samples obtained in winter (both sites) compared to the samples obtained in summer, these results are reported in Costabile et al. (2022) and in supplementary materials (Fig. S2).

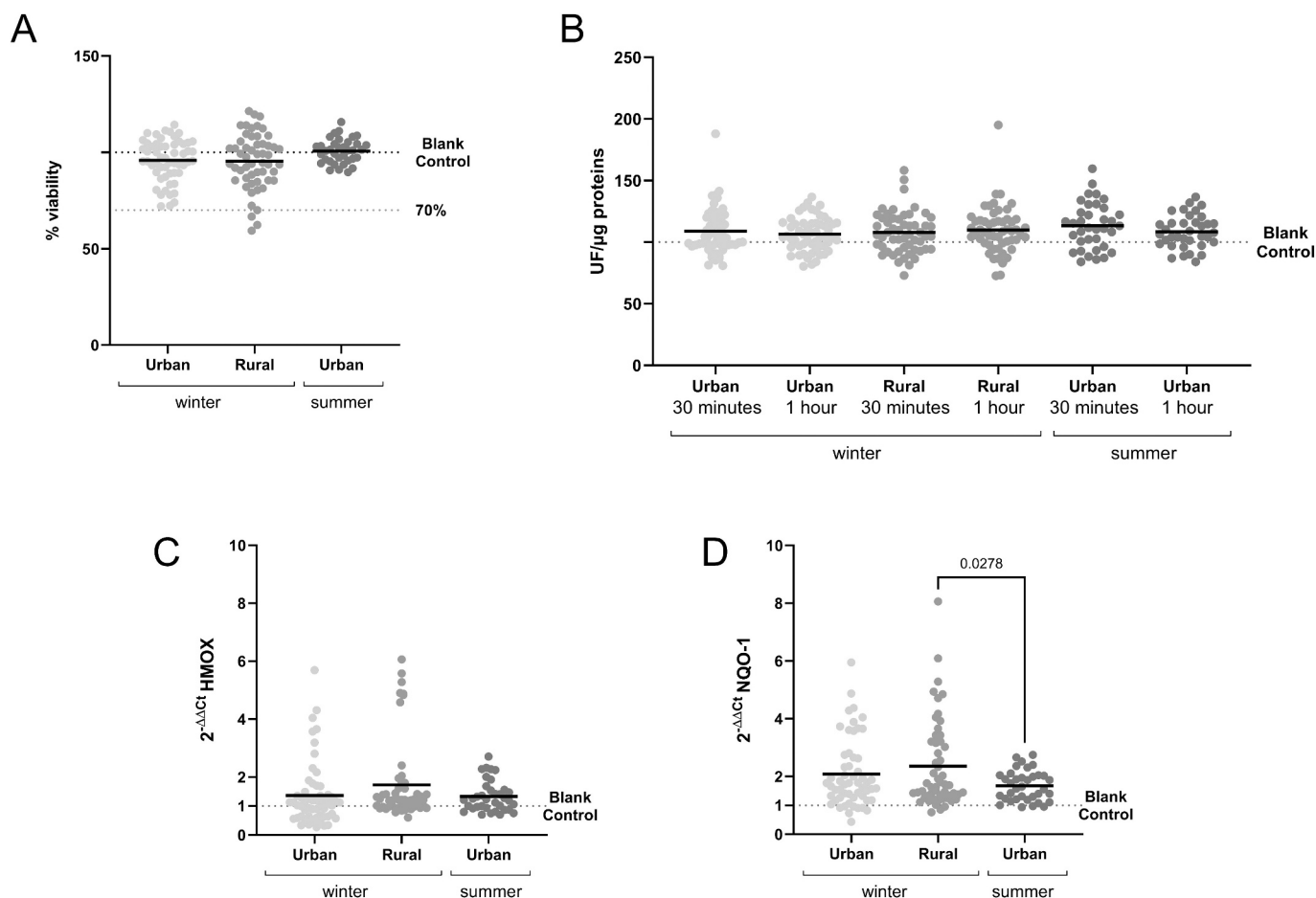
MUC5AC and ACE-2 resulted modulated in both direction with no differences among site and season (Fig. 5 B–C).

### 3.6. Correlation analysis

In Table 1 are reported the results of the Spearman correlation analysis between the toxicological and OP results (graph results can be found in the supplementary material, Fig. S3). Statistically significant positive correlations are found between:

- $OP^{DCFH}$  and the toxicological results obtained on IL-8 (BEAS-2B) secretion,  $\gamma$ -H2AX formation, and micronuclei formation.
- $OP^{AA}$  and the expression of CXCL-8, ACE-2.
- $OP^{DTT}$  and the expression of CXCL-8.
- $RP^{DPPH}$  and formation of  $\gamma$ -H2AX foci, and the expression of ATM, HMOX, and NQO-1.

Moreover, positive correlations are found between  $PM_{10}$  mass and the results of MTT assay and the expression of CXCL-8 and NQO-1, and between the  $PM_{10}$  mass concentration and the expression of NQO-1.



**Fig. 2.** Seasonal and site effect of PM<sub>1</sub> water-soluble fraction on cell viability and oxidative stress. BEAS-2B cells were exposed to 1:10 dilution of the extracts for 24 h (for cell viability and gene expression) or 30 min and 1 h (for DCFH-DA assay). (A) Average responses of exposed BEAS-2B cells on cell viability evaluated through MTT assay; data are expressed in percentage and compared to the blank control (BC = 100%). The 70% dashed line represents the 70% threshold of cell viability. (B) Average responses of exposed BEAS-2B cells on ROS formation, evaluated through DCFH-DA assay. Results are expressed in percentage, compared to blank control (100%), and normalized to the protein content. (C–D) Average response of exposed BEAS-2B cells in modulation of HMOX (C) and NQO-1 (D) gene expression. Results are reported as  $2^{-\Delta\Delta Ct}$ . Each dot represents a PM<sub>1</sub> water-soluble fraction from filter collected in different days. Statistical analysis was performed by One Way ANOVA followed by Tukey's Multiple Comparison test (only  $p$ -values < 0.05 are shown).

Negative correlations were found between:

- PM<sub>1</sub> mass concentration and ROS formation at 30 min.
- PM<sub>1</sub> mass and ROS formation at 30 min,  $\gamma$ -H2AX formation, and GADD45 $\alpha$  expression.
- OP<sup>DCFH</sup> and ROS formation at 30 min.
- OP<sup>AA</sup> and the  $\gamma$ -H2AX formation and the tail moment of the comet modified with ENDO III and FPG.
- OP<sup>DTT</sup> and IL-8 secretion in THP-1, micronuclei formation, and MUC5AC expression.
- OPQ<sup>DTT</sup> detected on quartz filter and micronuclei and  $\gamma$ -H2AX formation.
- RP<sup>DPPH</sup> and IL-8 secretion in THP-1.

#### 4. Discussion

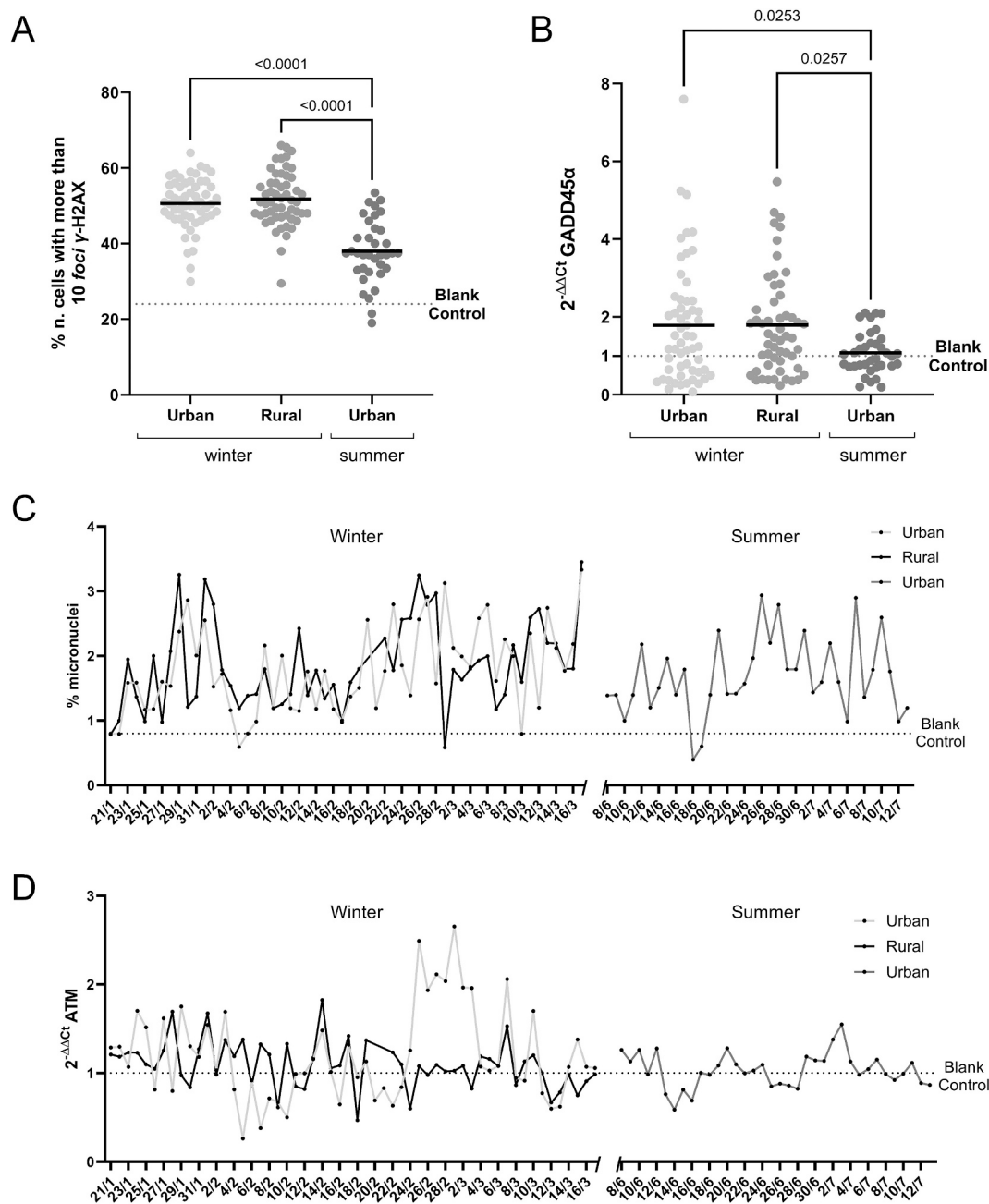
The present study aimed to evaluate and characterize the biological activity of PM<sub>1</sub> obtained on different days, sites and season in the Po Valley (Italy), and to investigate the reliability of OP as a predictive variable to define PM<sub>1</sub> biological activity (Costabile et al., 2022; Melzi et al., 2022). Differences were found among days, sites and season, indicating the sensitivity of the experimental model used to detect daily variation in PM<sub>1</sub>.

It was possible to observe the induction of DNA damage and

alteration of gene expression in absence of cytotoxicity, and at low level of PM<sub>1</sub> mass concentrations, with statistically significant differences between the two seasons evaluated.

Noticeably, no statistically significant differences in the markers of genotoxicity and ROS levels were observed between the two locations (urban vs. rural) in spite of the very different degree of anthropization and local emission intensity between the two sites. These observations indicate that the aerosol constituents driving the (water-soluble) PM<sub>1</sub> toxicity are well mixed at the regional scale, with little differences between urban and rural locations. Our results demonstrate that the reactive species of PM<sub>1</sub> can have residence times in the atmosphere long enough to largely spread over the whole Po Valley.

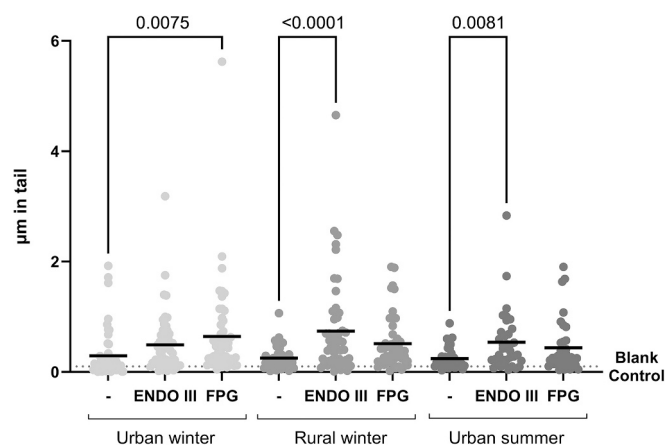
PM toxicity is widely described in literature and it is known that seasonal difference in composition of the PM can generate different toxicological responses, mainly described as induction of oxidative stress, inflammation and DNA damage (Abbas et al., 2019; Risom et al., 2005). Several studies have demonstrated the seasonal differences in PM toxicological response (Chen et al., 2018; Corsini et al., 2017; Longhin et al., 2013b). In the RHAPS campaign, statistically significant differences in toxicological responses were detected comparing winter and summer samples: stronger upregulation of NQO-1 and GADD45 $\alpha$ , increase of  $\gamma$ -H2AX foci formation, and higher IL-8 secretion in winter. Seasonal differences were detected also in THP-1, as shown by the results reported in Costabile et al. (2022), where the secretion was found



**Fig. 3.** Seasonal and site effects of PM<sub>1</sub> water-soluble fraction on  $\gamma$ -H2AX foci formation and DNA repair genes expression. BEAS-2B cells were exposed to 1:10 dilution of the extracts for 24 h. (A) Average responses on the formation of  $\gamma$ -H2AX foci evaluated through immunofluorescence; data are expressed in percentage of cells with >10 foci detected in the sample. The black dashed line represents the means of the blank controls (blank control = 24%). Statistical analysis was performed by One Way ANOVA followed by Turkey's Multiple Comparison test (only p-values < 0.05 are shown). (C) Daily responses on micronuclei formation. Results are expressed in percentage of micronuclei counted on the total cells of the sample (blank control = 0.8%, dashed line). (B and D) Average response in modulation of GADD45 $\alpha$  (B) and daily responses of ATM (D) genes expression. Results are reported as  $2^{-\Delta\Delta Ct}$ . Statistical analysis was performed by One Way ANOVA followed by Turkey's Multiple Comparison test (only p-values < 0.05 are shown).

to be higher in summer. In previous literature works, winter-derived PM<sub>2.5</sub> was shown to be more cytotoxic than the summer one collected in the same area (i.e., industrial area of Nanjing – China) (Chen et al., 2018). In this study, the average response of the samples did not show significant differences in cell viability between the two seasons analysed, even though it is possible to observe that in few winter samples cell viability decrease below the 70%. More importantly, statistically significant differences between IL-8 secretion in the two seasons analysed was observed. In our study, higher levels of IL-8 were detected in winter compared to summer in BEAS-2B, while higher levels were observed in THP-1 cells in summer, indicating that monocytes and lung

epithelial cells respond differently to PM components (Costabile et al., 2022). Similar results were found in a previous study, in which a higher release of IL-8 was found in summer-collected ambient air ultra-fine particles (UFPs) compared to winter UFPs in THP-1 cells (Corsini et al., 2017). This is also supported by other studies on PM<sub>10</sub> pro-inflammatory potential, which is found to be related to the period of sampling, with considerably more potent induction of IL-8 in summer (Camatini et al., 2012; Gualtieri et al., 2010). The differences in seasonal responses between THP-1 and BEAS-2B cells may be due to the possible enrichment of components of PM<sub>1</sub> derived from biological sources (i.e., spores and pollens). These components seem to have more effect on



**Fig. 4.** Seasonal and site effects of PM<sub>1</sub> water-soluble fraction on genotoxicity detected using the alkaline Comet assay modified with endonucleases (ENDOIII and FPG). BEAS-2B cells were exposed to 1:10 dilution of the extracts for 24 h. Average responses of exposed BEAS-2B cells on DNA damage presence, reported as mean of the tail moment values (in  $\mu\text{m}$ ). The treatment with the enzymes allows to detect the presence of oxidized bases. The blank dashed line represents the means of the blank controls (blank control = 0.1%). The different colours represent the use of the enzymes in modified comet assay. Statistical analysis was performed by Two Way ANOVA followed by Tukey's Multiple Comparison test (only p-values < 0.05 are shown).

THP-1 cells than BEAS-2B, since they are immune cells, and the same behaviour was previously demonstrated in more specific studies (Gualtieri et al., 2011; Longhin et al., 2013b).

Oxidative stress induction is one of the main effects related to PM exposure, reported in several studies (Comunian et al., 2020; Nozza et al., 2021; Peixoto et al., 2017; Valavanidis et al., 2008). In the current study, this noxious effect was more pronounced in winter than in summer, in particular regarding the expression of NQO-1, with a similar trend observed also for HMOX expression even though not statistically significant. Other studies have demonstrated that ROS activities and oxidative stress were substantially higher in the cold months compared to the warm months (Al Hanai et al., 2019; Hamad et al., 2015; Mirowsky et al., 2013). Seasonal differences were detected also in DNA damage induction, mainly in the analysis of  $\gamma$ -H2AX foci formation. In the literature, similar results were obtained with the comet assay and  $\gamma$ -H2AX evaluation after 24 h of PM treatment, that showed a significant increase of DNA damage especially in winter UFPs and PM<sub>2.5</sub> samples compared to control samples (Corsini et al., 2017; Niu et al., 2020). Genes investigated included those relevant for cell cycle regulation and DNA repair (ATM and GADD45 $\alpha$ ), resulted also mainly upregulated in winter when higher was the DNA damage observed. Only a few studies are available on the induction of DNA repair genes following PM exposure. It was shown that following isolation of leukocytes from healthy subjects exposed to PM, GADD45 $\alpha$  was up-regulated during winter and not during summer (Rossner et al., 2015). It has been reported that BEAS-2B exposed to PM<sub>2.5</sub> activate the ATM/CHK2 pathway, in accordance with the observed DNA damage (Gualtieri et al., 2011). Positive correlations were found between ATM expression and the expression of HMOX and NQO-1, and between HMOX and NQO-1 expression and DNA damage formation; probably those genes are activated in response to PM-induced oxidative stress also at DNA level, as previously suggested by Sánchez-Pérez et al. (2009). Oxidative stress is considered as an early event in the pathways triggered by PM, the mechanism by which ROS are formed is not completely described but the transition metals component of PM may contribute to ROS formation and DNA damage induction (Lakey et al., 2016). ACE-2 encoded protein is a functional receptor which belongs to angiotensin-converting enzyme family. It acts also as receptor for the spike glycoprotein of the SARS-CoV-2. Recent papers highlighted its overexpression in epithelial cells

also in presence of PM (Paital and Agrawal, 2021). MUC5AC encodes for one of the major lung mucus component and it has been shown to be regulated following PM exposure in normal human bronchial epithelial cells (Kim et al., 2017; Leclercq et al., 2016). For both these two genes we have shown the modulation following water-soluble PM exposure.

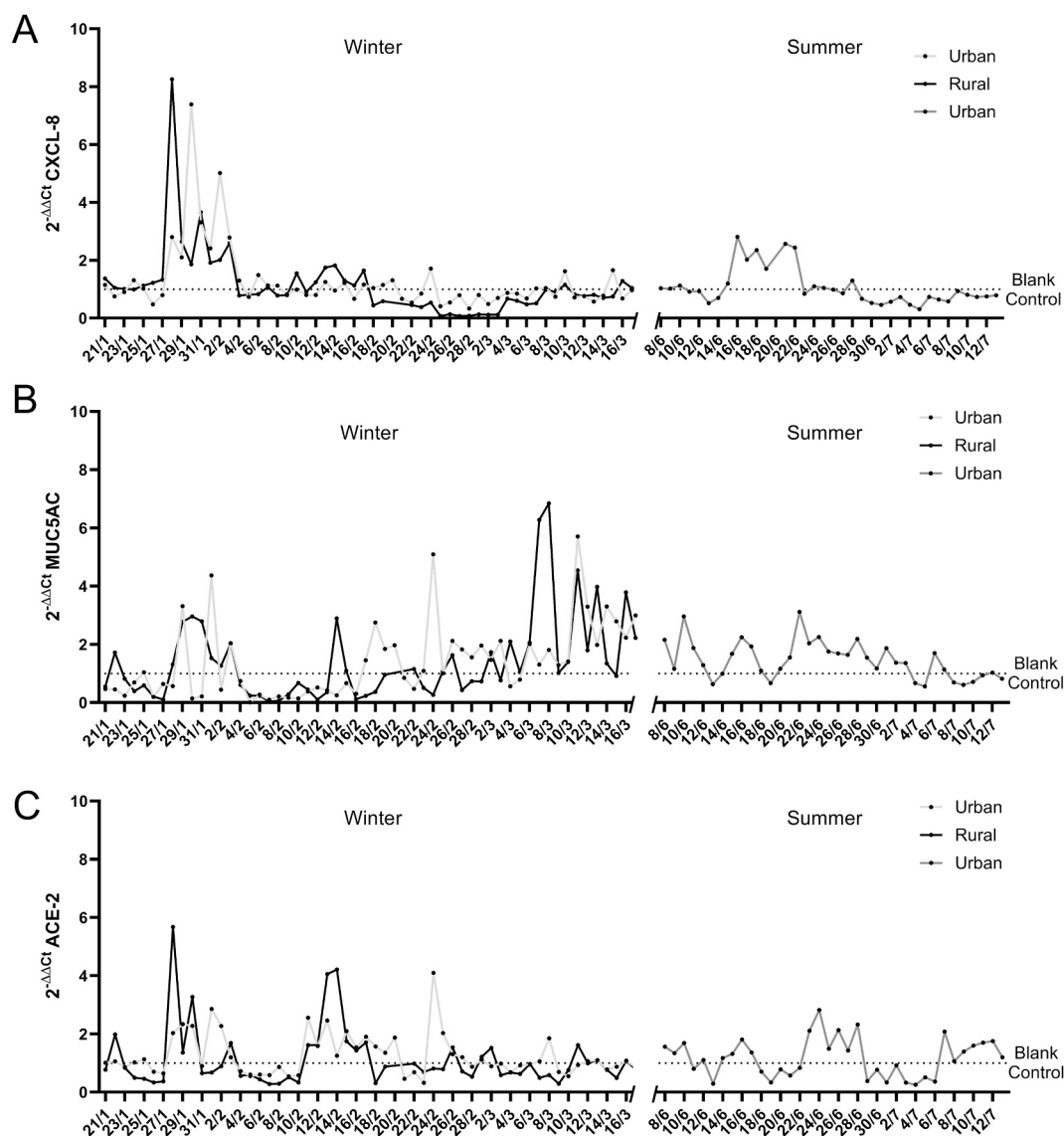
To date, very few studies have described the relationships between OP of particles and their toxicological effects. OP measurement is considered a promising and integrative method for estimating PM-induced health impacts. It is increasingly recognized that this metric is more closely associated with adverse health effects than ordinarily used PM mass concentration (Frezza et al., 2019). Thus, some authors suggest the integration of OP measurements along with the parameters currently evaluated in the air quality monitoring studies (Øvreik et al., 2015). Recently, associations were demonstrated with impairment to lung functions, increase admission in emergency department and OP characterization with AA and DTT methods (Fang et al., 2016; Janssen et al., 2015; Strak et al., 2012). We recently found that urban ambient aerosol high in total OP, as indicated by the DTT assay, with (possibly copper-containing) nanomaterials, showed higher pro-inflammatory and oxidative responses, this occurring at lower PM<sub>1</sub> concentrations (Costabile et al., 2023).

The presence of limited number of studies points out the difficulties in the identification of the more appropriate OP metric to be measured. Our results show seasonal variation in all the OP assays, with winter-OP values higher than summer-OP. Our correlation study demonstrated statistically significant correlation between the OP<sup>DCFH</sup> assays and oxidative stress, inflammation, DNA damage, HMOX and NQO-1 expression, IL-8 secretion in BEAS-2B,  $\gamma$ -H2AX foci formation and ATM expression. OP<sup>DCFH</sup> has been demonstrated to be sensitive toward fine particles with high content of organic compounds derived mainly by combustion processes (Jovanović et al., 2020; Perrone et al., 2016). Results obtained with this acellular assay (OP<sup>DCFH</sup>) were correlated to antioxidant cellular response (HMOX and NQO-1), inflammation, and double strands breaks formation.

Interestingly, also the evaluation of the presence of reducing species in PM samples by the DPPH assay showed positive correlations with toxicological responses of BEAS-2B exposed to water-soluble PM<sub>1</sub>, particularly for antioxidant response and DNA damage induction. The DPPH assay has been previously applied to test different types of PM components produced by specific emission sources and characterized by very different chemical compositions and it revealed the presence of reducing species in several components of atmospheric PM (mainly in urban dust, brake dust, and diesel dust) (Frezza et al., 2019). However, DPPH to evaluate the reducing properties of PM is a relatively new assay, and there is no standard experimental procedure. Therefore, at the present state of knowledge, the relevance of the information obtained from the DPPH assay has not yet been scientifically demonstrated, and further investigation is needed to deepen knowledge regarding the significance of the results obtained from this assay.

The observed negative correlations between biological parameters and OP measurements are more challenging to explain and somehow unexpected, and only speculations can be made, such as a different response toward different PM components. As correlation does not necessary means causality, additional studies are necessary to better understand the relationship between the different parameters measured. Although some of the correlations are positive and statistically significant in support of the hypothesis, most are still rather weak compared to an ideal correlation coefficient of 1. Therefore, there must still be other factors contributing to the overall variability in measured responses. Moreover, lack of correlation between some *in vitro* and OP results may also depends on the type of correlation analysis performed, which considered only one-to-one correlation, and there could be additive factors (or synergistic factors) among the variables. Moreover, the DTT assay does not seem to be affected by the type of membrane filter used, according to previous studies (Frezza et al., 2022), the lower extraction efficiency of the OP reactive species from quartz filters, compared to





**Fig. 5.** Seasonal and site effect of PM<sub>1</sub> water-soluble fraction on inflammation (CXCL-8) and MUC5AC and ACE-2 genes expression. BEAS-2B cells were exposed to 1:10 dilution of the extracts for 24 h. For gene expression, results are reported as  $2^{-\Delta\Delta C_t}$ . (A) Daily responses on CXCL-8 expression. (B–C) Daily response in modulation of MUC5AC (B) and ACE-2 (C) gene expression. Statistical analysis was performed by One Way ANOVA followed by Tukey's Multiple Comparison test (only p-values < 0.05 are shown).

PTFE filters, has already emerged a few times (Yang et al., 2014). In our recent study, we found an increased probability of toxicological markers for oxidative stress (HMOX) and inflammation (CXCL-8) in BEAS-2B cells exposed in ALI to PM<sub>1</sub> to be expressed at the very low doses of PM<sub>1</sub> exposure if these are accompanied by high concentrations of ROS-rich UFPs (Costabile et al., 2023). This could be associated with the enrichment in traffic-related nanoparticles of reactive compounds otherwise scavenged by larger particles. As larger particles are associated to higher PM<sub>1</sub> mass, the mechanism we proposed could provide a first explanation for the negative correlations here observed with PM<sub>1</sub>. Nevertheless, further studies should confirm this.

## 5. Conclusion

Owing to the correlation analysis of the results obtained from the same set of particles tested for OP and toxicological effects in a relevant cell target, our results provide useful biological support for evaluating the implementation of OP in air quality monitoring as a tool for estimating PM-induced toxicological responses, rather than PM mass

concentration, at least as far as concern the water-soluble component of PM.

## Acknowledgments/Fundings

We acknowledge the support of all the participants in the Redox-Activity and Health-Effects of Atmospheric Primary and Secondary Aerosol (RHAPS) project. This research was funded by the Italian Ministry of the University (MIUR), grant number 2017MSN7M8.

## CRediT authorship contribution statement

**Gloria Melzi:** Writing – original draft, Methodology, Investigation, Formal analysis. **Lorenzo Massimi:** Writing – original draft, Methodology, Investigation, Formal analysis. **Maria Agostina Frezzini:** Writing – original draft, Methodology, Investigation, Formal analysis. **Martina Iulini:** Methodology, Investigation, Formal analysis. **Naima Tarallo:** Methodology. **Matteo Rinaldi:** Writing – original draft, Methodology, Investigation, Formal analysis. **Marco Pagnione:** Writing – original

**Table 1**

Spearman correlation between the different toxicological assay and oxidative potential of the particles; the r-values are reported in the table, (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ). More analyses are available in the supplementary material.

|                | PM <sub>1</sub> Mass Conc. | PM <sub>1</sub> Mass | OP <sup>DCFH</sup> | OP <sup>AA</sup> | OP <sup>DTT</sup> | OPQ <sup>DTT</sup> | RP <sup>DPPH</sup> |
|----------------|----------------------------|----------------------|--------------------|------------------|-------------------|--------------------|--------------------|
| MTT            | 0.1243                     | 0.2104 *             | 0.1520             | 0.1056           | 0.0984            | 0.0269             | -0.0171            |
| ROS 30 min     | -0.2493 **                 | -0.2256 **           | -0.1808 *          | 0.0072           | -0.1353           | -0.1300            | -0.1363            |
| ROS 1 h        | -0.0489                    | -0.0145              | -0.0362            | -0.0847          | -0.0076           | -0.0787            | 0.0206             |
| IL-8 (BEAS-2B) | 0.1471                     | -0.0131              | 0.2848 **          | 0.0218           | 0.0808            | 0.0572             | 0.0919             |
| IL-8 (THP-1)   | -0.1144                    | 0.0552               | -0.0362            | 0.0059           | -0.2227 **        | -0.0543            | -0.3579 **         |
| Micronuclei    | -0.1127                    | -0.1283              | 0.1012             | -0.1205          | -0.1728 *         | -0.2612 *          | 0.0782             |
| γ-H2AX         | 0.0724                     | -0.1958 *            | 0.3777 **          | -0.1671 *        | 0.1061            | -0.2136 *          | 0.4301 **          |
| TM             | -0.1225                    | -0.1199              | -0.0323            | 0.0047           | -0.1191           | -0.1032            | -0.0075            |
| TM ENDOIII     | -0.0489                    | -0.0239              | 0.0755             | -0.2850 **       | -0.1274           | 0.0335             | -0.0978            |
| TM FPG         | -0.0026                    | -0.0474              | 0.0578             | -0.2343 **       | -0.1006           | 0.0613             | 0.0203             |
| ATM            | 0.1134                     | 0.0348               | 0.0082             | -0.1053          | 0.1018            | 0.0429             | 0.2593 **          |
| GADD45α        | -0.1347                    | -0.2945 **           | -0.0844            | -0.1448          | -0.0864           | -0.1050            | 0.0846             |
| HMOX           | 0.0764                     | 0.0435               | 0.0610             | -0.0849          | -0.0931           | 0.0046             | 0.1930 *           |
| CXCL-8         | 0.1592                     | 0.1946 *             | -0.1071            | 0.2178 **        | 0.2039 *          | 0.1488             | 0.0361             |
| NQO-1          | 0.2613 **                  | 0.2331 **            | 0.1435             | 0.0807           | 0.1563            | -0.0275            | 0.3207 **          |
| MUC5AC         | -0.0457                    | 0.0670               | 0.0104             | -0.0019          | -0.2157 *         | -0.1991            | 0.0244             |
| ACE-2          | 0.0748                     | 0.1050               | 0.0003             | 0.2125 *         | 0.0571            | 0.1777             | 0.0572             |

OP = oxidative potential; OPQ = oxidative potential on quartz filter; RP = reducing potential; OP data are normalized on sampling volume.

TM = Tail Moment.

draft, Methodology, Investigation. **Emma Nozza:** Methodology, Formal analysis. **Federica Crova:** Methodology, Investigation. **Sara Valentini:** Methodology, Investigation. **Gianluigi Valli:** Methodology, Investigation. **Francesca Costabile:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Silvia Canepari:** Supervision, Project administration, Conceptualization. **Stefano Decesari:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Roberta Vecchi:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Marina Marinovich:** Supervision, Resources, Project administration, Conceptualization. **Emanuela Corsini:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2024.116913>.

#### References

- Abbas, I., Badran, G., Verdin, A., Ledoux, F., Roumie, M., Lo Guidice, J.-M.M., Courcot, D., Garçon, G., 2019. In vitro evaluation of organic extractable matter from ambient PM<sub>2.5</sub> using human bronchial epithelial BEAS-2B cells: cytotoxicity, oxidative stress, pro-inflammatory response, genotoxicity, and cell cycle deregulation. *Environ. Res.* 171, 510–522. <https://doi.org/10.1016/j.envres.2019.01.052>.
- Al Hanai, A.H., Antkiewicz, D.S., Hemming, J.D.C., Shafer, M.M., Lai, A.M., Arhami, M., Hosseini, V., Schauer, J.J., 2019. Seasonal variations in the oxidative stress and inflammatory potential of PM<sub>2.5</sub> in Tehran using an alveolar macrophage model; the role of chemical composition and sources. *Environ. Int.* 123, 417–427. <https://doi.org/10.1016/j.envint.2018.12.023>.
- Barber, R.D., Harmer, D.W., Coleman, R.A., Clark, B.J., 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol. Genomics* 21, 389–395. <https://doi.org/10.1152/physiolgenomics.00025.2005>.
- Calas, A., Uzu, G., Kelly, F.J., Houdier, S., Martins, J.M.F., Thomas, F., Molton, F., Charron, A., Dunster, C., Oliete, A., Jacob, V., Besombes, J.-L., Chevri er, F., Jaffrezo, J.-L., 2018. Comparison between five acellular oxidative potential measurement assays performed with detailed chemistry on PM<sub>10</sub> samples from the city of Chamonix (France). *Atmos. Chem. Phys.* 18, 7863–7875. <https://doi.org/10.5194/acp-18-7863-2018>.
- Calas, A., Uzu, G., Besombes, J., Martins, J.M.F., Redaelli, M., Weber, S., Charron, A., Albinet, A., Chevri er, F., Brulfert, G., Mesbah, B., Favez, O., Jaffrezo, J., 2019. Seasonal variations and chemical predictors of oxidative potential (OP) of particulate matter (PM), for seven urban French sites. *Atmosphere (Basel)*. 10, 698. <https://doi.org/10.3390/atmos10110698>.
- Camatini, M., Corvaja, V., Pezzolato, E., Mantecca, P., Gualtieri, M., 2012. PM<sub>10</sub>-biogenic fraction drives the seasonal variation of proinflammatory response in A549 cells. *Environ. Toxicol.* 27, 63–73. <https://doi.org/10.1002/tox.20611>.
- Campbell, S.J., Uttinger, B., Lienhard, D.M., Paulson, S.E., Shen, J., Griffiths, P.T., Stell, A.C., Kalberer, M., 2019. Development of a physiologically relevant online chemical assay to quantify aerosol oxidative potential. *Anal. Chem.* 91, 13088–13095. <https://doi.org/10.1021/acs.analchem.9b03282>.
- Campbell, S.J., Wolfer, K., Uttinger, B., Westwood, J., Zhang, Z.-H., Bukowiecki, N., Steimer, S.S., Vu, T.V., Xu, J., Straw, N., Thomson, S., Elzein, A., Sun, Y., Liu, D., Li, L., Fu, P., Lewis, A.C., Harrison, R.M., Bloss, W.J., Loh, M., Miller, M.R., Shi, Z., Kalberer, M., 2021. Atmospheric conditions and composition that influence PM<sub>2.5</sub> oxidative potential in Beijing, China. *Atmos. Chem. Phys.* 21, 5549–5573. <https://doi.org/10.5194/acp-21-5549-2021>.
- Charrier, J.G., Anastasio, C., 2012. On dithiothreitol (DTT) as a measure of oxidative potential for ambient particles: evidence for the importance of soluble transition metals. *Atmos. Chem. Phys. Discuss.* 12, 11317–11350. <https://doi.org/10.5194/acpd-12-11317-2012>.
- Chen, Y., Luo, X.S., Zhao, Z., Chen, Q., Wu, D., Sun, X., Wu, L., Jin, L., 2018. Summer–winter differences of PM<sub>2.5</sub> toxicity to human alveolar epithelial cells (A549) and the roles of transition metals. *Ecotoxicol. Environ. Saf.* 165, 505–509. <https://doi.org/10.1016/j.ecoenv.2018.09.034>.
- Cho, A.K., Sioutas, C., Miguel, A.H., Kumagai, Y., Schmitz, D.A., Singh, M., Eiguren-Fernandez, A., Froines, J.R., 2005. Redox activity of airborne particulate matter at different sites in the Los Angeles Basin. *Environ. Res.* 99, 40–47. <https://doi.org/10.1016/j.envres.2005.01.003>.
- Comunian, S., Dongo, D., Milani, C., Palestini, P., 2020. Air pollution and COVID-19: the role of particulate matter in the spread and increase of COVID-19's morbidity and mortality. *Int. J. Environ. Res. Public Health* 17, 1–22. <https://doi.org/10.3390/ijerph17124487>.
- Cordelli, E., Bignami, M., Pacchierotti, F., 2021. Comet assay: a versatile but complex tool in genotoxicity testing. *Toxicol. Res. (Camb)*. 10, 68–78. <https://doi.org/10.1093/toxres/taaa093>.
- Corsini, E., Budello, S., Marabini, L., Galbiati, V., Piazzalunga, A., Barbieri, P., Cozzutto, S., Marinovich, M., Pitea, D., Galli, C.L., 2013. Comparison of wood smoke PM<sub>2.5</sub> obtained from the combustion of FIR and beech pellets on inflammation and DNA damage in A549 and THP-1 human cell lines. *Arch. Toxicol.* <https://doi.org/10.1007/s00204-013-1071-z>.
- Corsini, E., Vecchi, R., Marabini, L., Fermo, P., Becagli, S., Bernardoni, V., Caruso, D., Corbella, L., Dell'Acqua, M., Galli, C.L., Lonati, G., Ozgen, S., Papale, A., Signorini, S., Tardivo, R., Valli, G., Marinovich, M., 2017. The chemical composition of ultrafine particles and associated biological effects at an alpine town impacted by wood burning. *Sci. Total Environ.* 587–588, 223–231. <https://doi.org/10.1016/j.scitotenv.2017.02.125>.
- Corsini, E., Marinovich, M., Vecchi, R., 2019. Ultrafine particles from residential biomass combustion: a review on experimental data and toxicological response. *Int. J. Mol. Sci.* 20, 4992. <https://doi.org/10.3390/ijms20204992>.
- Costabile, F., Decesari, S., Vecchi, R., Lucarelli, F., Curci, G., Massab o, D., Rinaldi, M., Gualtieri, M., Corsini, E., Menegola, E., Canepari, S., Massimi, L., Argentini, S., Busetto, M., Di Iulio, G., Di Liberto, L., Paglione, M., Petenko, I., Russo, M.,

- Marinoni, A., Casasanta, G., Valentini, S., Bernardoni, V., Crova, F., Valli, G., Forello, A.C., Giardi, F., Nava, S., Pazzi, G., Prati, P., Vernocchi, V., La Torretta, T., Petralia, E., Stracquadanio, M., Zanini, G., Melzi, G., Nozza, E., Iulini, M., Caruso, D., Cioffi, L., Imperato, G., Giavarini, F., Battistoni, M., Di Renzo, F., Frezzini, M.A., Perrino, C., Facchini, M.C., 2022. On the redox-activity and health-effects of atmospheric primary and secondary aerosol: phenomenology. *Atmosphere (Basel)* 13 <https://doi.org/10.3390/atmos13050704>.
- Costabile, F., Gualtieri, M., Rinaldi, M., Canepari, S., Vecchi, R., Massimi, L., Di Iulio, G., Paglione, M., Di Liberto, L., Corsini, E., Facchini, M.C., Decesari, S., 2023. Exposure to urban nanoparticles at low PM1 concentrations as a source of oxidative stress and inflammation. *Sci. Rep.* 13, 18616. <https://doi.org/10.1038/s41598-023-45230-z>.
- Decesari, S., Sowlat, M.H., Hasheminassab, S., Sandrini, S., Gilardoni, S., Facchini, M.C., Fuzzi, S., Sioutas, C., 2017. Enhanced toxicity of aerosol in fog conditions in the Po Valley, Italy. *Atmos. Chem. Phys.* 17, 7721–7731. <https://doi.org/10.5194/acp-17-7721-2017>.
- EPA, 2024. US-EPA Air Quality Index [WWW Document]. URL. <https://www.epa.gov/criteria-air-pollutants/naaq5-table>.
- European Parliament, 2008. Directive 2008/50/EC of the European Parliament and of the Council on 21 May 2008 on Ambient Air Quality and Clearer Air in Europe.
- Fang, T., Verma, V., Bates, J.T., Abrams, J., Klein, M., Strickland, M.J., Sarnat, S.E., Chang, H.H., Mulholland, J.A., Tolbert, P.E., Russell, A.G., Weber, R.J., 2016. Oxidative potential of ambient water-soluble PM2.5 in the southeastern United States: contrasts in sources and health associations between ascorbic acid (AA) and dithiothreitol (DTT) assays. *Atmos. Chem. Phys.* 16, 3865–3879. <https://doi.org/10.5194/acp-16-3865-2016>.
- Fenech, M., 2000. The in vitro micronucleus technique. *Mutat. Res.* 455, 81–95. [https://doi.org/10.1016/S0027-5107\(00\)00665-8](https://doi.org/10.1016/S0027-5107(00)00665-8).
- Frezzini, M.A., Castellani, F., De Francesco, N., Ristorini, M., Canepari, S., 2019. Application of DPPH assay for assessment of particulate matter reducing properties. *Atmosphere (Basel)* 10, 816. <https://doi.org/10.3390/atmos10120816>.
- Frezzini, M.A., De Francesco, N., Massimi, L., Canepari, S., 2022. Effects of operating conditions on PM oxidative potential assays. *Atmos. Environ.* 268, 118802 <https://doi.org/10.1016/j.atmosenv.2021.118802>.
- Gerlier, D., Thomasset, N., 1986. Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods* 94, 57–63. [https://doi.org/10.1016/0022-1759\(86\)90215-2](https://doi.org/10.1016/0022-1759(86)90215-2).
- Gualtieri, M., Øvrevik, J., Holme, J.A., Perrone, M.G., Bolzacchini, E., Schwarze, P.E., Camatini, M., 2010. Differences in cytotoxicity versus pro-inflammatory potency of different PM fractions in human epithelial lung cells. *Toxicol. in Vitro* 24, 29–39. <https://doi.org/10.1016/j.tiv.2009.09.013>.
- Gualtieri, M., Øvrevik, J., Møller, P., Asare, N., Longhin, E., Dahlman, H.J., Camatini, M., Holme, J.A., 2011. Airborne urban particles (Milan winter-PM2.5) cause mitotic arrest and cell death: effects on DNA, mitochondria, AHR binding and spindle organization. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* 713, 18–31. <https://doi.org/10.1016/j.mrfmmm.2011.05.011>.
- Hamad, S.H., Shafer, M.M., Kadhim, A.K.H., Al-Omran, S.M., Schauer, J.J., 2015. Seasonal trends in the composition and ROS activity of fine particulate matter in Baghdad, Iraq. *Atmos. Environ.* 100, 102–110. <https://doi.org/10.1016/j.atmosenv.2014.10.043>.
- Hartwig, A., Arand, M., Epe, B., Guth, S., Jahnke, G., Lampen, A., Martus, H.J., Monien, B., Rietjens, I.M.C.M., Schmitz-Spanke, S., Schriever-Schwemmer, G., Steinberg, P., Eisenbrand, G., 2020. Mode of Action-Based Risk Assessment of Genotoxic Carcinogens. *Archives of Toxicology*. Springer, Berlin Heidelberg. <https://doi.org/10.1007/s00204-020-02733-2>.
- Janssen, N.A.H., Yang, A., Strak, M., Steenhof, M., Hellack, B., Gerlofs-Nijland, M.E., Kuhlbusch, T., Kelly, F., Harrison, R., Brunekreef, B., Hoek, G., Cassee, F., 2014. Oxidative potential of particulate matter collected at sites with different source characteristics. *Sci. Total Environ.* 472, 572–581. <https://doi.org/10.1016/j.scitotenv.2013.11.099>.
- Janssen, N.A.H., Strak, M., Yang, A., Hellack, B., Kelly, F.J., Kuhlbusch, T.A.J., Harrison, R.M., Brunekreef, B., Cassee, F.R., Steenhof, M., Hoek, G., 2015. Associations between three specific a-cellular measures of the oxidative potential of particulate matter and markers of acute airway and nasal inflammation in healthy volunteers. *Occup. Environ. Med.* 72, 49–56. <https://doi.org/10.1136/oemed-2014-102303>.
- Jovanović, M.V., Savić, J., Kovačević, R., Tasić, V., Todorović, Ž., Stevanović, S., Manojlović, D., Jovašević-Stojanović, M., 2020. Comparison of fine particulate matter level, chemical content and oxidative potential derived from two dissimilar urban environments. *Sci. Total Environ.* 708, 135209 <https://doi.org/10.1016/j.scitotenv.2019.135209>.
- Jung, M.H., Kim, H.R., Park, Y.J., Park, D.S., Chung, K.H., Oh, S.M., 2012. Genotoxic effects and oxidative stress induced by organic extracts of particulate matter (PM10) collected from a subway tunnel in Seoul, Korea. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* <https://doi.org/10.1016/j.mrgentox.2012.08.002>.
- Kim, H., Xue, X., 2020. Detection of Total reactive oxygen species in adherent cells by 2',7'-Dichlorodihydrofluorescein diacetate staining. *J. Vis. Exp.* <https://doi.org/10.3791/60682>.
- Kim, S.S., Kim, C.H., Kim, J.W., Kung, H.C., Park, T.W., Shin, Y.S., Kim, J.D., Ryu, S., Kim, W.J., Choi, Y.H., Song, K.S., 2017. Airborne particulate matter increases MUC5AC expression by downregulating Claudin-1 expression in human airway cells. *BMB Rep.* 50, 516–521. <https://doi.org/10.5483/BMBRep.2017.50.10.100>.
- Kramer, A.J., Rattanavaraha, W., Zhang, Z., Gold, A., Surratt, J.D., Lin, Y.-H., 2016. Assessing the oxidative potential of isoprene-derived epoxides and secondary organic aerosol. *Atmos. Environ.* 130, 211–218. <https://doi.org/10.1016/j.atmosenv.2015.10.018>.
- Lakey, P.S.J., Berkemeier, T., Tong, H., Arangio, A.M., Lucas, K., Pöschl, U., Shiraiwa, M., 2016. Chemical exposure-response relationship between air pollutants and reactive oxygen species in the human respiratory tract. *Sci. Rep.* 6, 32916. <https://doi.org/10.1038/srep32916>.
- Leclercq, B., Hapillon, 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. *Environ. Pollut.* 218, 1074–1088. <https://doi.org/10.1016/j.envpol.2016.08.059>.
- Li, N., Sioutas, C., Cho, A., Schmitz, D., Misra, C., Sempf, J., Wang, M., Oberley, T., Froines, J., Nel, A., 2003. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environ. Health Perspect.* 111, 455–460. <https://doi.org/10.1289/ehp.6000>.
- Longhin, Eleonora, Holme, J.A., Gutzkow, K.B., Arlt, V.M., Kucab, J.E., Camatini, M., Gualtieri, M., 2013a. Cell cycle alterations induced by urban PM2.5 in bronchial epithelial cells: characterization of the process and possible mechanisms involved. *Part. Fibre Toxicol.* 10, 1–19. <https://doi.org/10.1186/1743-8977-10-63>.
- Longhin, E., Pezzolato, E., Mantecca, P., Holme, J.A., Franzetti, A., Camatini, M., Gualtieri, M., 2013b. Season linked responses to fine and quasi-ultrafine Milan PM in cultured cells. *Toxicol. in Vitro* 27, 551–559. <https://doi.org/10.1016/j.tiv.2012.10.018>.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- 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, L.G., Marinovich, M., 2017. Ultrafine particles (UFPs) from domestic wood stoves: genotoxicity in human lung carcinoma A549 cells. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 820, 39–46. <https://doi.org/10.1016/j.mrgentox.2017.06.001>.
- Massimi, L., Ristorini, M., Simonetti, G., Frezzini, M.A., Astolfi, M.L., Canepari, S., 2020. Spatial mapping and size distribution of oxidative potential of particulate matter released by spatially disaggregated sources. *Environ. Pollut.* 266 <https://doi.org/10.1016/j.envpol.2020.115271>.
- Melzi, G., Nozza, E., Crova, F., Valentini, S., Forello, A., Valli, G., Vecchi, R., Costabile, F., Marinovich, M., Corsini, E., 2022. P07-30 study of the toxicological effect of winter and summer PM1 on BEAS-2B. *Toxicol. Lett.* 368, S131. <https://doi.org/10.1016/j.toxlet.2022.07.373>.
- Melzi, G., Nozza, E., Frezzini, M.A., Canepari, S., Vecchi, R., Cremonesi, L., Potenza, M., Marinovich, M., Corsini, E., 2023. Toxicological profile of PM from different sources in the bronchial epithelial cell line BEAS-2B. *Toxics* 11, 413. <https://doi.org/10.3390/toxics11050413>.
- Mirowsky, J., Hickey, C., Horton, L., Blaustein, M., Galdanes, K., Peltier, R.E., Chillrud, S., Chen, L.C., Ross, J., Nadas, A., Lippmann, M., Gordon, T., 2013. The effect of particle size, location and season on the toxicity of urban and rural particulate matter. *Inhal. Toxicol.* 25, 747–757. <https://doi.org/10.3109/08958378.2013.846443>.
- Møller, P., Danielsen, P.H., Karotki, D.G., Jantzen, K., Roursgaard, M., Klingberg, H., Jensen, D.M., Christophersen, D.V., Hemmingsen, J.G., Cao, Y., Loft, S., 2014. Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles. *Mutat. Res. Rev. Mutat. Res.* 762, 133–166. <https://doi.org/10.1016/j.mrrev.2014.09.001>.
- Møller, P., Scholten, R.H., Roursgaard, M., Kraus, A.M., 2020. Inflammation, oxidative stress and genotoxicity responses to biodiesel emissions in cultured mammalian cells and animals. *Crit. Rev. Toxicol.* 50, 383–401. <https://doi.org/10.1080/10408444.2020.1762541>.
- Niu, B.Y., Li, W.K., Li, J.S., Hong, Q.H., Khodahemmati, S., Gao, J.F., Zhou, Z.X., 2020. Effects of DNA damage and oxidative stress in human bronchial epithelial cells exposed to PM2.5 from Beijing, China, in winter. *Int. J. Environ. Res. Public Health* 17, 1–14. <https://doi.org/10.3390/ijerph17134874>.
- Nozza, E., Melzi, G., Marabini, L., Marinovich, M., Piazzza, S., Khalilpour, S., Dell'Agli, M., Sangiovanni, E., Dell'Agli, M., Sangiovanni, E., 2020. Rhus coriaria L. Fruit Extract Prevents UV-A-Induced Genotoxicity and Oxidative Injury in Human Microvascular Endothelial Cells. *Antioxidants* 9, 292. <https://doi.org/10.3390/antiox9040292>.
- Nozza, E., Valentini, S., Melzi, G., Vecchi, R., Corsini, E., 2021. Advances on the immunotoxicity of outdoor particulate matter: a focus on physical and chemical properties and respiratory defence mechanisms. *Sci. Total Environ.* 780, 146391 <https://doi.org/10.1016/j.scitotenv.2021.146391>.
- Orru, H., Ebi, K.L., Forsberg, B., 2017. The interplay of climate change and air pollution on health. *Curr. Environ. Heal. Reports* 4, 504–513. <https://doi.org/10.1007/s40572-017-0168-6>.
- Øvrevik, J., Refsnes, M., Låg, M., Holme, J., Schwarze, P., 2015. Activation of Proinflammatory responses in cells of the airway mucosa by particulate matter: oxidant- and non-oxidant-mediated triggering mechanisms. *Biomolecules* 5, 1399–1440. <https://doi.org/10.3390/biom5031399>.
- Paital, B., Agrawal, P.K., 2021. Air pollution by NO2 and PM2.5 explains COVID-19 infection severity by overexpression of angiotensin-converting enzyme 2 in respiratory cells: a review. *Environ. Chem. Lett.* 19, 25–42. <https://doi.org/10.1007/s10311-020-01091-w>.
- Panina, Y., Germond, A., Masui, S., Watanabe, T.M., 2018. Validation of common housekeeping genes as reference for qPCR gene expression analysis during iPSC reprogramming process. *Sci. Rep.* 8, 8716. <https://doi.org/10.1038/s41598-018-26707-8>.
- Park, M., Jo, H.S., Lee, K., Jang, M., Kim, S.D., Kim, I., Borlaza, L.J.S., Lim, H., Shin, H., Chung, K.H., Choi, Y.-H.H., Park, S.G., Bae, M.-S.S., Lee, J., Song, H., Park, K., 2018.

- Differential toxicities of fine particulate matters from various sources. *Sci. Rep.* 8, 17007. <https://doi.org/10.1038/s41598-018-35398-0>.
- Peixoto, M.S., de Oliveira Galvao, M.F., Batistuzzo de Medeiros, S.R., 2017. Cell death pathways of particulate matter toxicity. *Chemosphere* 188, 32–48. <https://doi.org/10.1016/j.chemosphere.2017.08.076>.
- Perrone, M.G., Zhou, J., Malandrino, M., Sangiorgi, G., Rizzi, C., Ferrero, L., Dommen, J., Bolzacchini, E., 2016. PM chemical composition and oxidative potential of the soluble fraction of particles at two sites in the urban area of Milan, northern Italy. *Atmos. Environ.* 128, 104–113. <https://doi.org/10.1016/j.atmosenv.2015.12.040>.
- Risom, L., Møller, P., Loft, S., 2005. Oxidative stress-induced DNA damage by particulate air pollution. *Mutat. Res.* 592, 119–137. <https://doi.org/10.1016/j.mrfmmm.2005.06.012>.
- Rossner, P., Tulupova, E., Rossnerova, A., Libalova, H., Honkova, K., Gmuender, H., Pastorkova, A., Svecova, V., Topinka, J., Sram, R.J., 2015. Reduced gene expression levels after chronic exposure to high concentrations of air pollutants. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* 780, 60–70. <https://doi.org/10.1016/j.mrfmmm.2015.08.001>.
- Sánchez-Pérez, Y., Chirino, Y.I., Osormio-Vargas, Á.R., Morales-Bárceñas, R., Gutiérrez-Ruiz, C., Vázquez-López, I., García-Cuellar, C.M., 2009. DNA damage response of A549 cells treated with particulate matter (PM10) of urban air pollutants. *Cancer Lett.* 278, 192–200. <https://doi.org/10.1016/j.canlet.2009.01.010>.
- Singh, N.P., McCoy, M.T., Tice, R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175, 184–191. [https://doi.org/10.1016/0014-4827\(88\)90265-0](https://doi.org/10.1016/0014-4827(88)90265-0).
- Stafoggia, M., Oftedal, B., Chen, J., Rodopoulou, S., Renzi, M., Atkinson, R.W., Bauwelinck, M., Klompmaker, J.O., Mehta, A., Vienneau, D., Andersen, Z.J., Bellander, T., Brandt, J., Cesaroni, G., de Hoogh, K., Fecht, D., Gulliver, J., Hertel, O., Hoffmann, B., Hvidtfeldt, U.A., Jöckel, K.-H., Jørgensen, J.T., Katsouyanni, K., Ketzel, M., Kristoffersen, D.T., Lager, A., Leander, K., Liu, S., Ljungman, P.L.S., Nagel, G., Pershagen, G., Peters, A., Raaschou-Nielsen, O., Rizzuto, D., Schramm, S., Schwarze, P.E., Severi, G., Sigsgaard, T., Strak, M., van der Schouw, Y.T., Verschuren, M., Weinmayr, G., Wolf, K., Zitt, E., Samoli, E., Forastiere, F., Brunekreef, B., Hoek, G., Janssen, N.A.H., 2022. Long-term exposure to low ambient air pollution concentrations and mortality among 28 million people: results from seven large European cohorts within the ELAPSE project. *Lancet Planet. Heal.* 6, e9–e18. [https://doi.org/10.1016/S2542-5196\(21\)00277-1](https://doi.org/10.1016/S2542-5196(21)00277-1).
- Strak, M., Janssen, N.A., Godri, K.J., Gosens, I., Mudway, I.S., Cassee, F.R., Lebret, E., Kelly, F.J., Harrison, R.M., Brunekreef, B., Steenhof, M., Hoek, G., 2012. Respiratory health effects of airborne particulate matter: the role of particle size, composition, and oxidative potential—the RAPTES project. *Environ. Health Perspect.* 120, 1183–1189. <https://doi.org/10.1289/ehp.1104389>.
- 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. *Environ. Mol. Mutagen.* 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).
- Valavanidis, A., Fiotakis, K., Vlachogianni, T., 2008. Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms. *J. Environ. Sci. Heal. - Part C Environ. Carcinog. Ecotoxicol. Rev.* 26, 339–362. <https://doi.org/10.1080/10590500802494538>.
- Vecchi, R., Marazzan, G., Valli, G., 2007. A study on nighttime-daytime PM10 concentration and elemental composition in relation to atmospheric dispersion in the urban area of Milan (Italy). *Atmos. Environ.* 41, 2136–2144. <https://doi.org/10.1016/j.atmosenv.2006.10.069>.
- Velali, E., Papachristou, E., Pantazaki, A., Choli-Papadopoulou, T., Planou, S., Kouras, A., Manoli, E., Besis, A., Voutsas, D., Samara, C., 2016. Redox activity and in vitro bioactivity of the water-soluble fraction of urban particulate matter in relation to particle size and chemical composition. *Environ. Pollut.* 208, 774–786. <https://doi.org/10.1016/j.envpol.2015.10.058>.
- Verma, V., Ning, Z., Cho, A.K., Schauer, J.J., Shafer, M.M., Sioutas, C., 2009. Redox activity of urban quasi-ultrafine particles from primary and secondary sources. *Atmos. Environ.* 43, 6360–6368. <https://doi.org/10.1016/j.atmosenv.2009.09.019>.
- Verma, V., Fang, T., Xu, L., Peltier, R.E., Russell, A.G., Ng, N.L., Weber, R.J., 2015. Organic aerosols associated with the generation of reactive oxygen species (ROS) by water-soluble PM2.5. *Environ. Sci. Technol.* <https://doi.org/10.1021/es505577w>.
- World Health Organization, 2021. WHO Global Air Quality Guidelines 1–360.
- Yang, A., Jedynska, A., Hellack, B., Kooter, I., Hoek, G., Brunekreef, B., Kuhlbusch, T.A.J., Cassee, F.R., Janssen, N.A.H., 2014. Measurement of the oxidative potential of PM2.5 and its constituents: the effect of extraction solvent and filter type. *Atmos. Environ.* 83, 35–42. <https://doi.org/10.1016/j.atmosenv.2013.10.049>.
- Yang, A., Janssen, N.A.H., Brunekreef, B., Cassee, F.R., Hoek, G., Gehring, U., 2016. Children's respiratory health and oxidative potential of PM 2.5: the PIAMA birth cohort study. *Occup. Environ. Med.* 73, 154–160. <https://doi.org/10.1136/oemed-2015-103175>.