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(Article begins on next page)

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Evaluating the genotoxicity of urban PM2.5 using PCR-based methods in human lung cells and the Salmonella TA98 reverse test

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Abstract A number of compounds found in particulate matter with an aerodynamic diameter <2.5 (PM2.5) can interact with DNA either directly or after enzymatic transformation to induce DNA modifications. These particulate matter (PM)-induced alterations in DNA may be associated with increased frequencies of pollution-associated diseases, such as lung cancer. In the present study, we applied different methods to assess the mutagenicity and genotoxicity of monthly PM2.5 organic extracts collected over a full year. We used the Salmonella assay, exposed cultured human embryonic lung fibroblasts and applied extracellular lactate dehydrogenase (LDH) and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) assays to assess the cytotoxicity of PM2.5 on the cells. We assessed both the expression levels of a number of DNA repair genes (using qRT-qPCR) and the genetic profile of the treated cells compared to the control. The expression levels of XRCC1 and APE1, which are involved in the first steps of base excision repair, as well as ERCC1, XPA and XPF, which encode nucleotide excision repair subunits, were analysed. The monthly mean of the PM2.5 collected was 35.16±22.06 μg/m$_3$. The mutagenicity of PM2.5 to TA98 was 46±50 net revertants/m$_3$, while the mutagenicity to TA98+S9 was 17±19 net revertants/m$_3$. The mean IC$_{50}$ values were 2.741±1.414 and 3.219±2.764 m$_3$ of equivalent air in the XTT and LDH assays, respectively. A marked and significant increase in APE1 expression levels was observed in the exposed cells. This effect was also significantly correlated with mutagenicity ($p<0.01$). No induced AFLP fragment profile alterations were detected. The proposed approach seems to be useful for integrated evaluation and for highlighting the mechanisms inducing DNA damage.

Introduction

Urban areas, particularly megacities, are the most polluted (Jain and Khare 2008; Nagdeve 2006; Zhang et al. 2008), along with areas with significant emission points and/or close valleys in which there is a lack of pollution dispersion (Nemery et al. 2001; Pope 1996; Ta et al. 2004; Voigt et al. 1998). Worldwide, several epidemiological studies have revealed morbidity and mortality increases following particle exposure. Fine particles are particularly dangerous (Eftim et al. 2008; Kappos et al. 2004; Krewski et al. 2005; Pope 2000). Particulate matter (PM) pollution is often measured using indicators, such as the concentration of dispersed particulate matter with an aerodynamic diameter <10 μm (PM10) and particulate matter with an aerodynamic diameter <2.5 (PM2.5) (Krzyzanowski 2008). The aerodynamic diameter of PM is inversely correlated with its ability to enter into the respiratory tree and to arrive at the alveolar level, at which blood-air exchange occurs (Sorensen et al. 2003a, b). The adverse health effects of PM include the exacerbation of asthma (Viera et al. 2009; Zhang et al. 2009), chronic obstructive pulmonary disease (COPD) (Lindgren et al. 2009) and death fromcardiovascular disease (Mills et al. 2009; Nogueira 2009) and lung cancer (Beelen et al. 2008; Grant 2009; Vineis et al. 2007). The mechanisms driving these effects are not completely understood, but they are thought to involve the direct effects of organic toxins and oxidative stress, followed by inflammation (Moller et al. 2008).

The correlation between air pollution and cancer has been widely discussed in epidemiological terms (Valavanidis et al. 2008). Cohort studies have shown that populations exposed to high levels of air pollution have a 10–30 % greater risk of developing lung cancer than populations exposed to low levels of air pollution (Kam et al. 2013; Vinikoor-Imler et al. 2011); however, the contributions of confounding factors, particularly in studies of European populations, are debatable (Gallus et al. 2008).

In October 2013, the IARC declared that outdoor air pollution was the leading environmental cause of cancer death and classified this agent as a human carcinogen. Moreover, particulate matter, as a major component of outdoor pollution, was also classified as a group 1 agent (Loomis et al. 2013).

Molecular studies have demonstrated that DNA damage was specifically correlated with fine PMpollution (Alfaro-Moreno et al. 2002; de Kok et al. 2005;Mehta et al. 2008). The effects of PM pollution on DNA can range from the formation of bulky DNA adducts or point mutations to single- or doublestrand breaks that result in genetic and chromosomal instability. Early effects are normally corrected by repair mechanisms, but these repair mechanisms may fail during aging or disease processes. Some genotoxic agents not only damage the integrity of the genome but also directly or indirectly affect gene

expression. These genotoxic effects may increase the incidence of different types of cancer and, in the long term, may result in decreased genetic integrity in exposed populations (Beelen et al. 2008; Brunekreef et al. 2009).

In recent years, molecular genetics studies have demonstrated both structural changes, such as genetic instability, and regulatory changes, including epigenetic phenomena (Ji and Hershey 2012).Avariety of polymerase chain reaction (PCR)-based techniques are currently available to evaluate the frequency of induced DNA alterations in fragment profiles, expressed as number of lost and introduced fragments for each exposure in comparison to the control, all of which involve the random or semi-random amplification of genomic DNA (Atienzar and Jha 2006; Baurand et al. 2013; Ben Salem et al. 2014; Srut et al. 2013; Vardar et al. 2014). Induced variations in the nucleotide sequence are the result of a complex equilibriumbetween the genetic response to environmental stressors, DNA repair systems and DNA damage resulting from the stressors. DNA repair systems include the base excision repair (BER) and nucleotide excision repair (NER) systems (Wood et al. 2005). These systems can be affected by urban particulate exposure (Libalova et al. 2012; Mukherjee et al. 2013). Fine PMis a complex mixture of compounds that have both antagonistic and synergistic effects, making the application of in vitro assays relevant when evaluating a global effect on a biologic model (Pfuhler et al. 2013).

In the present study, we assessed the results of parallel in vitro methods applied to the analysis of cells exposed to urban PM2.5 organic extracts collected in Turin, one of the most polluted cities in Europe (Traversi et al. 2011). These methods included the following assays: Salmonella test to assess the mutagenic potential of PM2.5, lactate dehydrogenase (LDH) and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) to assess the cytotoxic potential, real-time quantitative polymerase chain reaction (RT-qPCR) expression to assess the modulation of DNA repair proteins and amplified fragment length polymorphism (AFLP) to measure the induced DNA alterations in fragment profiles.

Materials and methods

The complete study design, from the sampling of the environmental PM2.5 to the early effect tests, is illustrated in Fig. 1. The assays include the Salmonella test and cytotoxicity, AFLP and gene expression assays in human embryonic lung fibroblasts (HELFs). The aim of such test choice was to use a classic mutagenic test as Salmonella test and to place side by side, using the same samples, various tests on human cells to observe the following: cytotoxicity, gene expression modulations and DNA alterations in fragment profiles.

PM2.5 sampling and organic extraction

PM2.5 was collected from January to December 2008 in a meteorological-chemical station located in a background urban area in southern Turin using a sampler (Analitica Strumenti, Pesaro, Italy), according to directive UNIEN14907. PM2.5 (PM passing through a size-selective inlet with 50 % efficiency cutoff at an aerodynamic diameter of 2.5 μM) was collected on glass micro-fibre filters (Type A/E, 8″×10″, PALL Corporation, 25 Harbor Park Drive, Port Washington, NY 11050, USA) at a flow rate of approximately 500 l/min. The samples were collected over a 24-h period, and sample duration was controlled by a timer that was accurate to ±15 min. The exact flow was calculated daily and corrected for variations in atmospheric pressure and actual differential pressure across the filter. The filters were conditioned for 48 h and weighed using an analytical balance (±10 μg) before and after sampling to calculate the concentration of PM in the air sampled. The procedures were conducted according to the European Committee for Standardisation (CEN 1998). The sampling station was located in an urban area that was not directly exposed to any relevant emission source; therefore, the PMconcentration was representative of the exposure level of the resident population, without accounting for specific traffic-related or industrial exposure.

Each day, the collected filters were pooled to obtain a unique month-long organic sample. Extractions of each pooled sample were performed using at least 80 cycles of a Soxhlet apparatus (BUCHI B-811, Savatec, Torino, Italy)with acetone (product number 439126 Sigma-Aldrich), followed by evaporation induced by a Rotavapor (Savatec, Torino, Italy) instrument and resuspension of the sample with dimethyl-3-sulfoxide (DMSO, product number 13409023 Sigma-Aldrich) to obtain an equivalent concentration of 0.2 m3/μl.

We chose the organic extraction because in the literature, it showed most of the genotoxicity (Claxton et al. 2004; Claxton and Woodall 2007). The doses for the following assays were defined as equivalent PM2.5 organic extract corresponding to a cubic metre of inhalable air to describe real human exposure conditions during a year of exposure to urban air pollution (Maruyama et al. 2006). Moreover, it was possible to evaluate the dose corresponding to PM2.5 mass (Supplementary Material 1) and the effect per mass unit of PM2.5, as shown in Fig. 2.

Salmonella reversion test

Mutagenicity assays were performed for each of the monthly DMSO extracts, according to the classic procedure. Increasing doses of the PM2.5 organic extract (5, 10, 20 and 50 μl of the 0.2 m3/μl DMSO suspension) were tested. All experiments were performed in triplicate. The slope of the dose–response curve (revertants/m3) was calculated by the least squares

linear regression from the first linear portion of the dose–response curve (Gilli et al. 2007). The results are displayed as net revertants per cubic metre (rev/m3), but the net revertants per microgram of PM2.5 are also presented. The mutagenic activity of airborne particulate extracts was studied using Salmonella typhimurium strain TA98, both in the presence and absence of Aroclor-induced rat-liver homogenate activation (S9). The inclusion of a mammalian liver homogenate ensured that some of the potential carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), were converted into their active forms by liver enzyme systems (Claxton et al. 2004). The number of spontaneous revertants obtained during the bioassay sessions were 18±2 and 23±6 for S. typhimurium strain TA98 in the absence and presence of S9 mix, respectively.

Cell exposures

Low-passage human embryonic lung fibroblast (HELF) cells were grown as a monolayer in minimum essential medium (MEM) (M2279 Sigma-Aldrich) containing 10 % foetal bovine serum (FBS) (N4762 Sigma-Aldrich), 4 mM L-glutamine, 2 mM sodium pyruvate and 100 µg/ml of penicillin/streptomycin at 37 °C in a humidified atmosphere of 5%CO2. Cytotoxicity was evaluated using both an extracellular lactate dehydrogenase (LDH) assay and an assay measuring mitochondrial dehydrogenase activity in living cells by cleaving formazan to a tetrazolium salt (XTT). The experiments were performed according to the instructions provided in the Cytotoxicity Kit in terms of number of technical repeats (at least 3), plate size (96-well plates), cell number (20,000 cells/well), positive and negative controls, duration of the exposition and acceptability of variation (In CytoTox, PAN I, Xenometrix) using different concentrations of the PM2.5 organic extracts. Doses ranging from 0.25 % (0.5 m3 of equivalent air) to 3 % (6 m3 of equivalent air) were tested and corresponded to the equivalent doses shown in Supplementary Material 1. The results for the two assays are expressed as the equivalent air (in m3 per ml) required to observe 50 % of the effect (IC50). Moreover, the results for the PM2.5 mass unit are also shown.

RNA and DNA extractions

HELF cells were cultured at densities of 5×106 cells/plate in 90-mm cell culture plates. Six plates were exposed to each sample, and six plates were used as controls. Two plates were exposed to each of the three concentrations of PM2.5 organic extracts (1, 2 and 3 %), and two plates were exposed only to the organic solvent DMSO and served as controls (1, 2 and 3 %). We performed two replicates. After 72 h of incubation with the PM2.5 organic extracts, we isolated pure genomic DNA (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma-Aldrich, GN70) from the three different exposure doses for each sample and control. In addition, we extracted RNA (GenElute Mammalian total RNA Miniprep kit, Sigma-

Aldrich, RTN70) from the other plates for the three different exposure doses for each sample and control. The concentration and quality of the nucleic acid extracts was tested using spectrophotometric analyses, as outlined in the extraction kits. The DNA extracts were stored at −20 °C prior to the AFLP analyses, and the RNA extracts were stored at −80 °C prior to the retrotranscription reactions.

Gene expression analysis

Reverse transcription of total RNA for all monthly samples was achieved using the High Capacity cDNA Archive Kit (iScript cDNA Synthesis Kit, Bio-Rad). APE1, XRCC1, XPA, ERCC1 and XPF gene expression levels were evaluated using a PCR master mix (SsoFast EvaGreen Supermix, Bio-Rad) and the Chomo 4 Real-time PCR system (Bio-Rad) with OpticonMonitor 3.0 software. The thermal protocol consisted of enzymatic activation at 98 °C for 30 s, followed by 40 denaturation cycles at 95 °C for 10 s, annealing/extension at 60 °C for 30 s and a plate read. After all cycles had been completed, a melt curve was generated at 65–95 °C (in 0.5 °C increments). The primers for each expression target are shown in Table 1 (Arase et al. 2002; Chang et al. 1999; Silva et al. 2008). The relative changes in gene expression were calculated using the ΔΔCt method (Livak and Schmittgen 2001), taking into account both the control expression levels and normalisation against the endogenous housekeeping genes, HPRTI and GAPDH, as suggested in the literature (Vandesompele et al. 2002). The results are expressed as the fold increase in the expression following exposure to each dose (1, 2 and 3 %, which corresponded to 2, 4 and 6 m3/ml, respectively).

AFLP analyses

AFLP is a DNA-fingerprinting technique (Vos et al. 1995). In this technique, a set of distinct DNA fragments is generated from a single DNA sample using PCR after DNA digestion with restriction enzymes and adapter ligation. These fragments are then separated on a high-resolution denaturing polyacrylamide gel, producing a complex electrophoretic profile. AFLP was conducted using a commercial kit (Invitrogen AFLPAnalysis System I and Starter Primer Kit), according to the manufacturer's specifications and a previously described protocol (Vos et al. 1995). We used this technique to detect induced DNA alterations in AFLP fragment profiles in the DNA extracts of the cells that had been exposed to 3 % doses compared to the controls exposed only for the winter months. We used the amplification reaction profile that was described in the kit manual.

This cell line has greater genomic stability than transformed cells, as do other embryonic cells (Bylund et al. 2004). This high genomic stability is critical for experiments assessing induced DNA alterations in AFLP fragment profiles and was verified by the extraction and amplification of DNA

from two different vials of cells that were frozen from the same cell culture aliquot, stored in liquid nitrogen and thawed on different days.

The DNA amplification products were analysed by separating an aliquot of each reaction on 5 % denaturing polyacrylamide gels in TBE electrophoresis buffer for 3 h at 100 W. Bands were visualised using silver staining (Bassam and Gresshoff 2007). The polymorphic bands were scored by visual inspection of the stained gels. The frequency of observed DNA alterations in AFLP fragment profiles was calculated as the ratio of the number of polymorphic bands to the total number of recorded bands and is reported as a percentage.

Statistics

The local Environmental Protection Agency (ARPA Piemonte) produced an air pollution data set (ARPA Piemonte PdT 2008) that examined PM10 and PM2.5 (collected in the sampling station), including benzo(a)pyrene and total PAHs, with the addition of 1, 2Aand 2B PAH, according to the IARC assessment (Belpomme et al. 2007). These data were included in the experimental database. Statistical analyses were performed using the IBM SPSS Package, version 21.0, for Windows. Analysis of variance (ANOVA) was used to compare the means of the variables using Tukey's post hoc test, and Spearman's rank-order correlation coefficient was used to assess the relationships between the variables. The difference between two means was considered to be significant at $p<0.05$. Probit linear regression was used to calculate the IC50 values for the cytotoxicity tests. Seasonal analyses were performed. December, January and February were considered to be the winter months; March, April and May were considered to be the spring months; June, July and August were considered to be the summermonths; and September, October and November were considered to be the autumn months

Results and discussion

Environmental levels and mutagenicity of the PM2.5

The PM2.5 concentration ranged from 15.41 to 83.71 μg/m3, as described in Table 2. The annual mean was well above the reference levels for the protection of human health, which were established to be 20 μg/m3 by the EU and 10 μg/m3 by the WHO (EEA 2012). The concentrations of PM2.5 displayed a seasonal trend, with higher concentrations in the winter and autumn. All seasonal mean comparisons were significant: winter, 62.32±16.42; spring, 18.72±3.32; summer, 16.57±1.10; and autumn, 43.01 15.96 (ANOVA, $F=31.645$, $p<0.001$). The annual ratio of PM2.5 to PM10 was 0.69, revealing the prevalence of fine particles that is typical of urban locations.

The mutagenicity incidence was sevenfold that of spontaneous revertants, but generally, it was not high, particularly in

the presence of S9 enzyme-mediated activation (Table 2). The mutagenicity observed in the absence of metabolic activation was greater, highlighting the fact that direct mutagens play major roles in mutagenic processes. Generally, the contribution of S9 mix tomutagen activation is quite relevant (Claxton et al. 2004). However, this contribution, which is imputable mainly to the polycyclic aromatic hydrocarbons (PAHs), can vary consistently based on the sampling site (Claxton and Woodal l 2007) . The annual mean of the PM2.5 benzo(a)pyrene and PAHs were 0.6 and 3.1 ng/m3, respectively (ARPA Piemonte PdT 2008). The correlation between PAHs and mutagenicity was statistically significant (Spearman's rho 0.870, $p<0.01$). Such compounds may play roles in the mutagenic potential of PM2.5. Moreover, Turin and southern Europe are characterised by the presence of directly mutagenic nitro compounds, such as the nitro-PAHs (Traversi et al. 2011), which are more often mutagenic and carcinogenic than PAH derivatives (Albinet et al. 2008).

As for the gravimetric data, themutagenicity observedwith the Salmonella assay differed in a seasonal manner ($F=25.144$, $p<0.001$ for the TA98 strain and $F=34.888$, $p<0.001$ for the TA98 strain with S9 mix), with the exception of summer versus spring (Fig. 2a). When comparing the PM2.5 level and mutagenicity in the absence and presence of S9 mix, the calculated Spearman's rho was 0.846 ($p=0.001$) and 0.825 ($p=0.001$), respectively. The mutagenic responses with and without metabolic activation were correlated (Spearman's rho 0.993, $p<0.001$). Moreover, when mutagenicity was expressed as net revertants per microgram of PM2.5, valuable responses were observed, particularly during the months of January, February, November and December (Fig. 2a). This evidence confirms not only that PM2.5 concentration is greater in winter, but also the mutagenicity per mass unit is also higher, likely because of alterations in the chemical composition of the mixture of particles, as previously discussed (Traversi et al. 2011). In contrast, other types of genotoxic effects, such as oxidative stress, may be higher during the spring or other seasons (Bastonini et al. 2011).

The observed results are comparable to previous studies in which data regarding the mutagenic properties of polluted urban areas in Europe in general and Southern Europe in particular were collected (Cassoni et al. 2004; Claxton et al. 2004; Claxton and Woodall 2007).

The year 2013 has been designated by theWHO as the year of air, in reference to the serious economic problems caused by air pollution and the impact of aero-dispersed pollution on human health (WHO-Europe 2013). Some estimates indicate that the incidence of lung cancer increases 8 % for every 10 μg/m3 increase in PM2.5 levels in the air above the background level. It is assumed that the threshold below which no harmful effects are observed is equal to approximately

5 μg of PM2.5/m3 (WHO 2005). The annual mean level of PM2.5 is above this reference concentration in all southern European regions, particularly in urban areas. This finding was confirmed in Turin during our sampling period, despite the fact that the annual mean PM2.5 levels have markedly decreased over the past few years (ARPA Piemonte SP, CSI Piemonte 2014; WHO 2005).

Cytotoxicity

Cytotoxic effects were observed in HELF cells. A dosedependent response was observed, and the mean monthly IC50 was equal to 2.741±1.414 and 3.219±2.764 m3 of equivalent air/millilitre for the XTT and LDH assays, respectively, which corresponds to approximately 96 and 113 μg, respectively, of aero-dispersed PM2.5. The maximum effect on mitochondrial activity of the samples reached 80% of total inhibition for every sample but was particularly high during spring and the first few months of summer. The cytotoxic effects were also affected by different seasonal environmental mixtures (ANOVA, $F=92.044$, $p<0.001$ for LDH, where a significantly higher IC50 was observed during autumn than during the other seasons; $F=27.937$, $p<0.001$ for XTT, where the IC50 values during autumn and winter differed significantly from those during summer and spring) (Fig. 2b). The two IC50 cytotoxicity evaluations were significantly correlated (Spearman's rho, 0.622; $p<0.001$). In this case, the observed effect on the cells was more relevant because of both the higher quantity and the higher toxicity of the PM2.5 composition, as evidenced by the data analysis per unit mass of exposure (Fig. 2b, secondary x–y axis).

Plasma membrane damage, as assessed bymeasuring LDH release, is comparable to the effect recorded in other studies, in which the LDH increase was approximately two times the effect of the control at a dose of 200 μg/ml of PM10 and in which a major effect was observed for the finer PM size fractions (Happo et al. 2010). The mitochondrial respiratory chain effects, as measured using XTT reduction, in the current study were similar to those reported in the literature when lung cells were used as a biological model of exposure to various particles (Breznan et al. 2013). The cytotoxic effects were relevant, and the results were sub-lethal for the cells at levels greater than 2 m3 of equivalent air/millilitre, corresponding to 70 μg of PM2.5 mass.

Gene expression

Gene expression analysis revealed onlymild modulation (modulation of APE1 expression was only evident at the lowest exposure dose). Dose-dependent effects on levels of expression were not observed. However, we examined sub-lethal exposure doses, as revealed by the previously described cytotoxicity tests. At the higher doses (2 and 3 %), the cellular gene expression machinery may become completely disordered. APE1 and XRCC1 are genes involved in the first steps of

BER after the recognition of damaged bases by specific DNA glycosylases, incision of the affected DNA strand by an apurinic/apyrimidinic (AP) endonuclease and subsequent repair involving the XRCC1 protein. APE1 was clearly upregulated (Table 2), particularly following exposure to non-lethal doses (i.e. lower than the IC50 values identified in the cytotoxic tests); however, only modest and non-significant XRCC1 modulation was observed after the first dose was applied (Table 3). Recently, other authors observed that nanoparticles in particular were leading to defective XRCC1 DNA repair, proliferation arrest and inflammatory responses. The reduced changes with respect to the expected effects seemed to be caused by the capability of the particle to adsorb cytosolic proteins on its surface (Asharani et al. 2012).

After recognition of the damaged base by the appropriate DNA glycosylase, the glycosylase creates an apurinic or apyrimidinic site (AP site). The DNA backbone is then cleaved by a DNA AP endonuclease (APE1). Its activity creates a single-stranded DNA nick 5′ to the AP site, as opposed to the nick being created 3′ to the AP site as a result of DNA AP lyase activity. APE1 was constitutively upregulated, and the ANOVA analysis of APE1 expression was significant when the first dose was considered in respect to the other doses ($F=4.983$, $p=0.013$), which were either lethal or displayed cytotoxic effects that were too high to reveal evidence of gene expression modulation.

NER is the main DNA repair pathway involved in the repair of UV- and carcinogen-induced DNA damage in mammalian cells and is highly conserved in nature. DNA damage is first recognised by XPA, but our results revealed that modulation of this target was not an effective marker, because the mean observed expression was lower than control levels (Table 2). When the first dose was considered with regard to the others, the expression level differences were significant (ANOVA, $F=3.364$, $p=0.048$). Other authors recently demonstrated that XPA expression was not clearly increased when studying itsmodulation following benzo(a)pyrene exposure in human bronchial epithelial cells (Yang et al. 2013). The regulation of ERCC1 and XPF expression is quite similar. During the DNA repair process, ERCC1 and XPF form a complex and are involved in the incision of the 3′ side of the damage, resulting in the removal of a piece of DNA that is approximately 29 nucleotides in length. Our data indicated that ERCC1 and XPF are significantly correlated based on the expression data obtained following exposure to the first dose. Constant and moderate increases in their expression were observed, equating to approximately 20–30 % (Table 3). Similarly, the induction of relevant genes involved in DNA repair and apoptosis, and ERCC1 induction specifically, has been reported after exposure to PM10 (Bastonini et al. 2011).

To correlate the gene expression results with the Salmonella

assay data and PM2.5 levels (Table 4), we used only the data obtained from the two cytotoxicity tests following exposure to the lowest dose (the dose that was below the IC50 value), which were expressed as cubic metre of equivalent air per square centimetr of cell culture.

Interestingly, we observed a significant and proportional correlation between APE1 overexpression and the mutagenicity observed in the Salmonella assay that was induced by monthly samples (Table 4). A proportional effect was observed both in base insertions into a bacterial cell in the S. typhimurium TA98 strain and in the necessity of removing AP sites in human lung fibroblasts.

AFLP

Our AFLP analyses were performed on the DNA extracts that were derived from cell cultures that had been exposed to the highest dose from the winter months. No lost or introduced fragments were observed. Recently, a study of the effects of exposure to benzo(a)pyrene and ethyl methanesulfonate revealed that the percentage of lost or introduced fragments (compared to the control) was 12 % (Srut et al. 2013). Furthermore, it may be inferred that an effect that results from exposure to high concentrations of a single chemical compound is more repeatable than an effect observed after exposure to environmental mixtures, such as the PM2.5 urban organic extracts.

It has been suggested that 100 % reproducibility of AFLP fingerprints may be achieved by excluding bands with intensities <1 % of the combined intensity of all bands and by excluding the largest and the smallest bands, which account for 10 % of the total recorded bands (Bagley et al. 2001). However, this approach can be improved in terms of resolution and repeatability using an automatic method for the detection of the AFLP fragments with respect to the visual inspection of the stained gels.

Conclusions

The process of scientific investigation can be used to determine the level of exposure to mutagenic and genotoxic contaminants and to define an appropriate protection factor for human health. A threshold effect should also be evaluated, considering individual susceptibility and also the susceptibility specific to some population groups, such as children, elderly patients, patients with predisposing polymorphisms and patients with previous pathologies.

The screening methods that are used for genotoxic assessments are generally based on consolidated protocols, such as the Salmonella assay in bacteria and the Comet assay in human cells. These methods provide a useful model for assessing the ability of these environmentalmixtures to induce genetic and chromosomal damage in the exposed cells (Eastmond et al. 2009). After this damage has occurred, however, it is not possible to define the nature and chromosomal

location of the induced damage. PCR-based methods may be a tool to address this problem. Although the PCR-based techniques are promising, many problems remain, including low reproducibility and the lack of standardisation (Bustin et al. 2009). Moreover, other kinds ofmethods able to analyse a great number of genes are now available such as microarray.
In the present study, mutagenicity, cytotoxicity and gene expression assays were applied to a panel of the same samples. Overall, the results revealed the capacity of PM2.5 to produce DNA alterations and support genotoxic processes. Interesting correlations between damage processes that were similar from a biologic point of view were observed, such as point mutations in Salmonella and increases in AP site removal necessity in HELF cells. On the other hand, the dose of exposure is a crucial point, and careful consideration must be given to distinguish a dose that is able to produce a detectable effect from a lethal dose. An extension of the endpoints investigated, including epigenetic targets, may help to clarify the complex carcinogenic pathways activated by exposure to PM2.5.

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Authors' contributions Dr. Traversi designed the study and performed the PM2.5 sample extractions, Salmonella, cytotoxicity and gene expression assays. Dr. Cervella performed the AFLP assay. Dr. Gilli provided the PM2.5 samples and contacted the local Environmental Protection Agency.Dr. Gilli and Dr. Traversi analysed the data.Dr. Traversi prepared the manuscript. All authors approved the final manuscript.

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Keywords Particulate matter · DNA damage · Mutagenicity · Gene expression · AFLP · Urban air pollution

Abbreviations

AFLP	Amplified fragment length polymorphism
BER	Base excision repair
LDH	Extracellular lactate dehydrogenase
NER	Nucleotide excision repair
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
PM	Particulate matter
PM10	Particulate matter with an aerodynamic diameter <10 μm
PM2.5	Particulate matter with an aerodynamic diameter <2.5 μm
RT-qPCR	Real-time quantitative polymerase chain reaction
XTT	2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt

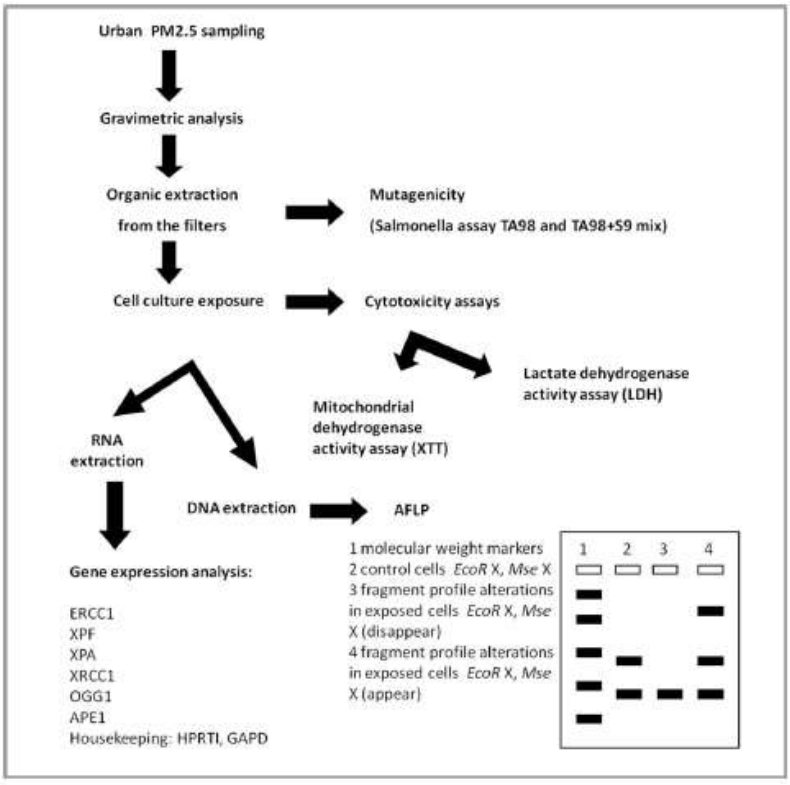

Fig. 1 An overview of the study design from the PM2.5 sampling to the expected biological responses. After PM2.5 was collected on filters, the mass was evaluated. Organic extraction was then performed. The extracts were subdivided and used for mutagenicity evaluations in bacteria and for cytotoxicity and DNA alteration evaluations in HELF cells. The cytotoxicity assays included the MTT and LDH assays, while the genotoxic assays included both gene expression analyses and AFLP. The last step shows the appearance or disappearance of a band on the polyacrylamide gel

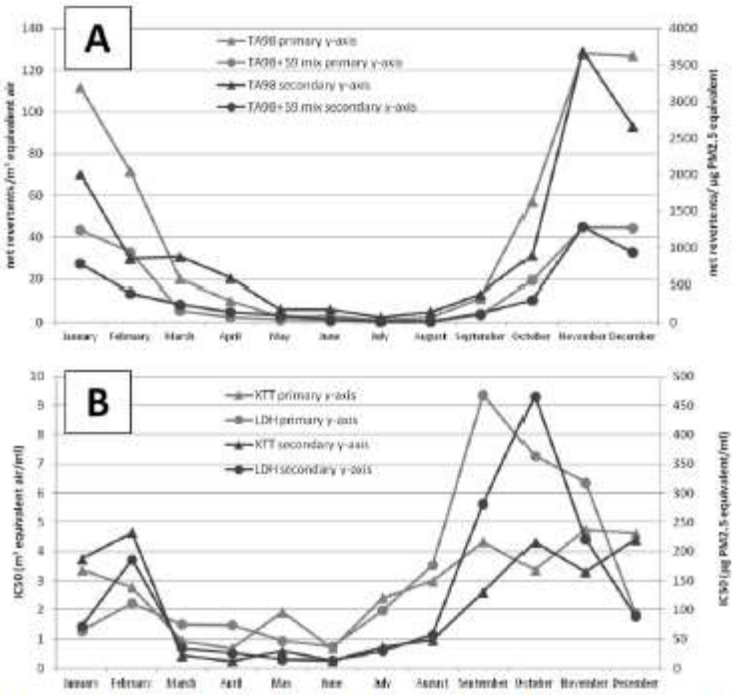

Fig. 2 Mutagenic and cytotoxic effects observed by month. Seasonal analysis of the data collected is subdivided into winter, autumn, spring and summer means and standard deviations. The seasonal ANOVA for this data series is significant ($p < 0.01$). **a** Mutagenicity data resulting from a Salmonella assay that was conducted with *Salmonella typhimurium* TA98 in the presence and absence of S9 mix. The data are expressed on the primary ordinate axis as net revertants/cubic meter of equivalent air and as net revertants/microgram of PM2.5 on the secondary ordinate axis. **b** Cytotoxicity data resulting from the LDH and XTT assays. The data are expressed on the ordinate axis as the IC_{50} as cubic meter of equivalent air and as the IC_{50} as microgram of PM2.5 on the secondary ordinate axis

Table 1 Primer details for the gene-expression assays, including target gene functions and references

Gene function during DNA repair	Primers	5′- sequence - 3′	Reference
Hypoxanthine phosphoribosyl-transferase 1 pathway, purine synthesis in salvage	HPRTI_f HPRTI_r	TGACACTGGCAAAACAATGCA GGTCCTTTTCACCAGCAAGCT	Vandesompele et al. 2002
Glyceraldehyde-3 phosphate dehydrogenase, oxidoreductase in glycolysis and gluconeogenesis	GAPD_f GAPD_r	TGCACCACCAACTGCTTAGC GGCATGGACTGTGGTCATGAG	
Involved in incision on 3′ side of damage; forms complex with XPF	ERCC1_f ERCC1_r	GGGAATTTGGCGACGTAATTC GCGGAGGCTGAGGAACAG	Chang et al. 1999
The XPF with ERCC1 cuts the DNA on either side of the damage. The result is the removal of a piece of DNA	XPF_f XPF_r	TGACCCTAGCAAGCCTTTCTCTCT GGGGCACCAGAGAATCCGTAGTC	
Xeroderma pigmentosum group A, damage recognition	XPA_f XPA_r	GAGTTTCAACCCAGGTCTTCTCATTTT AATGGTAACAGTGAGTAGTAGTAGTGC	
DNA-repair protein, X-ray repair complementing defective repair	XRCC1_f XRCC1_r	GCAAACCCCGAGGAGAAGGCA ACTGCTGGAACCTGGCCCTGC	Arase et al. 2002
AP endonucelase, incision of the affected DNA strand	APE1_f APE1_r	GCGGCAGCGGAAGAC GCCTCCTTCTCAGTTTTCTTTGCT	Silva et al. 2008

Table 2 Descriptive analysis of the data collected after gravimetric examination, Salmonella assays and cytotoxicity tests (XTT and LDH)

Target	Min	Max	Mean	Standard deviation
PM2,5 (μg/m^3)	15.41	83.71	35.16	22.06
Salmonella typhimurium TA98 (net revertants/m^3)	1	128	46	50
Salmonella typhimurium TA98+S9 (net revertants/m^3)	0	45	17	19
XTT IC_{50} (m^3 equivalent air/ml)	0.696	4.752	2.741	1.414
LDH IC_{50} (m^3 equivalent air/ml)	0.762	9.360	3.219	2.764
ERCC1 (fold increase)	0.84	2.10	1.28	0.32
XPA (fold increase)	0.05	3.85	0.91	0.78
XPF (fold increase)	0.77	2.69	1.31	0.39
XRCC1 (fold increase)	0.50	2.10	1.17	0.36
APE1 (fold increase)	0.75	5.89	2.08	1.43

The results are expressed as monthly mean during a full year of sampling

Table 3 Modulation of gene expression by month and evaluation of the effects of PM2.5 1 % dose on targeted gene expression after considering the control and after normalising the expression with respect to house-keeping genes

Sample	ERCC1	XPA	XPF	XRCC	APE1
January	1.58	0.24	1.69	1.68	5.24
February	2.10	1.12	2.69	1.04	5.89
March	1.17	1.05	0.94	1.20	2.36
April	1.10	0.76	1.25	0.94	2.48
May	1.09	3.85	1.04	1.20	0.77
June	0.86	1.65	1.32	0.92	1.19
July	1.17	0.92	1.40	1.13	0.95
August	1.08	0.38	1.17	1.37	1.32
September	1.34	1.90	1.03	0.89	2.81
October	1.59	2.69	1.58	1.71	5.53
November	1.85	1.14	2.10	1.39	5.25
December	0.97	0.24	1.24	0.50	2.43
Mean	1.33	1.33	1.46	1.16	3.02
Standard deviation	0.38	1.07	0.51	0.34	1.93

Table 4 Spearman's rho correlation between mutagenic (*Salmonella typhimurium* net revertant/m^3 without and with S9 mix) and genotoxic biological evaluations (forder increase of expression after exposure 1 % exposure equivalent to 20 m^3 of equivalent air for plate)

	TA98	TA98+S9	ERCC1	XPA	XPF	XRCC1	APE1
TA98		0.993**	ns	ns	ns	ns	0.755**
TA98+S9			ns	ns	ns	ns	0.741**
ERCC1				ns	0.608*	ns	0.783**
XPA					ns	ns	ns
XPF						ns	0.594*
XRCC1							ns

ns not significant

* significant at $p<0.05$, ** highly significant at $p<0.01$