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Particulate matter toxicity and health effects: *in vitro* assessment of the mechanisms of action

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Contents

Abstract

Urban airborne particulate matter (PM) is known to increase morbidity and mortality due to cardiopulmonary diseases related to inflammatory processes and genotoxic effects. The study of PM-induced toxicity represents a very important field in order to understand the clinical outcomes and define the most harmful components involved. Despite the researchers' effort, there are still unresolved questions regarding the cell mechanisms inducing the different adverse effects. Moreover, which PM components are more significant in determining the biological responses is still a debated question, although this aspect is of primary importance for the individuation of the most impacting sources, which have to be regulated. Thus, the aims of this thesis were

- the analysis of the biological effects induced by PM exposure in cell lines representative of the lung apparatus, to evaluate the role of season, location and size of particles
- the understanding of the mechanisms and molecular pathways activated by Milan PM fractions with particular attention to winter PM2.5, linking the outcomes to the particles physical and chemical properties.

The results obtained demonstrated that the PM-induced biological effects were related to the site and season of sampling, and directly linked to the specific PM chemical composition. The PMs size was another significant factor in eliciting the toxic potential, not only for the chemical composition of particles of diverse dimension, but also for a different bioavailability of the compounds adsorbed, which is higher for the finest fractions. In particular, summer PM10 resulted to be the most cytotoxic and pro-inflammatory fraction for its high content of endotoxins and metals, known inducers of both these biological effects. Winter PMs, especially the fine

ones (PM2.5, PM1 and PM0.4), produced a genotoxic effect and caused alterations of the cell cycle through the induction of DNA damage and acting as mitotic spindle poisons. Organic components, in particular PAHs, were responsible of such effects through ROS production and CYP enzymes-mediated reactive molecules formation. A peculiar aspect evidenced was the formation of abnormal mitotic spindles, and in particular the presence of tripolar spindles, which were able to satisfy the spindle assembly checkpoint and perform cell division.

The data reported provide a significant contribution to the knowledge of PM toxicity, describing biological processes that can be involved in the clinical and epidemiological observations widely reported; however a complete understanding of the mechanisms of action at cell level still lacks. A further insight in the comprehension of PM health impact may derive by further investigations on different cell lines, cultures of primary pulmonary cells and by the assembly of an alveolar-capillary barrier for the study of the particles systemic effects.

Abbreviations

α-NF	α-naphtoflavone
ACS	American Cancer Society
AED	Aerodynamic equivalent diameter
AM	Alveolar macrophages
APC	Anaphase-promoting complex
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BaP	Benzo[a]pyrene
CB	Carbon black
CDK	Cyclin-dependent kinase
COPD	Chronic obstructive pulmonary disease
DCFH-DA	2'7'-dichlorodihydrofluorescein diacetate
DEG	Differentially expressed genes
DEP	Diesel exhaust particle
DSB	Double strand break
EC	Elemental carbon
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
HPLC-FD	High-performance liquid chromatography-fluorescence detection
IL	Interleukine
LAL	Limulus Amebocyte Lysate
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M/A	Metaphase/anaphase
MN	Micronuclei
MT	Microtubule
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
OC	Organic carbon
PAH	Polycyclic aromatic hydrocarbon
PCD	Programmed cell death
PM	Particulate matter
ROS	Reactive oxygen species
RNS	Reactive nitrogen species

SAC	Spindle assembly checkpoint
SCGE	Single cell gel electrophoresis
SEM	Scanning electron microscopy
SSB	Single strand break
TC	Total carbon
TEM	Transmission electron microscopy
Thio	Thiourea
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOT	Thermal optical transmission
TSP	Total suspended particulate
UFP	Ultrafine particle
WHO	World Health Organization
XRF	X-ray fluorescence

List of papers

This thesis is based on the experimental work presented in the papers and supplementary data listed below:

1. Introduction

According to WHO air quality guidelines (last global update, 2005) each year more than 2 million premature deaths can be attributed to the effects of urban outdoor and indoor air pollution. One of the most dangerous air pollutants is particulate matter (PM), which represents a mixture of solid and liquid particles suspended in the air, which vary in size, composition and origin. For PM, a quantitative relationship between the concentration of the pollutant monitored in ambient and specific health outcomes as mortality and hospitalizations for respiratory and cardiovascular diseases has been reported (Brunekreef and Forsberg, 2005; Pope and Dockery, 2006).

Despite intensive research to identify the biological mechanisms explaining these associations, there are still unresolved issues. In this perspective, detailed *in vitro* studies are highly needed and, concordantly, the work here presented has the general aim to provide new elements to understand PM adverse effects.

1.1. *PM physico-chemical characteristics*

PM is an air-suspended mixture of solid and liquid particles that vary in number, size, shape, surface area, chemical composition, solubility, and origin (Pope and Dockery, 2006).

1.1.1. *Size*

Among all these characteristics, the size is the one used for classification due to its intrinsic importance: in fact the atmospheric deposition rates of particles, their residence times in the atmosphere and deposition patterns within the lung are strongly influenced by particles size.

Since atmospheric particles are not spherical, their size is described by the aerodynamic equivalent diameter (AED) i.e. the diameter of a sphere of

density of 1 g/cm^3 that would have the same physical behavior when suspended in the air.

The Total Suspended Particulate (TSP) consists of all particles that remain suspended in the atmosphere and have a diameter ranging from less than 10 nm up to 100 μm. In the years, different methods or conventions have been developed to classify particles by size: 1) modes, based on the observed size distributions and formation mechanisms; 2) occupational health sizes, based on the entrance into various compartments of the respiratory system; 3) cut point, based on the 50% cut point of the specific sampling device (EPA, 2004).

1) Whitby (1978) was the first one to propose the modal classification based on size distribution of TSP, describing a three modal pattern represented by the coarse particles mode (peak 5-30 μm), the accumulation mode (peak 0.15-0.5 μm) and the Aitken nuclei range or nuclei mode (peak 0.015-0.04 μm). Afterwards, a four modal distribution has been proposed (Fig. 1; EPA, 2004) dividing the nucleation region, or ultrafine particles (< 10 nm), and the Aitken (nuclei) region (10 to 100 nm).

The selected modes reflect different generation sources, which determine size and composition of particles; the coarse particles are produced by mechanical and resuspension processes, the accumulation range, also named fine particles, results mainly from the coagulation of smaller particles, condensation of volatile species, gas-to-particle conversion processes and from finely ground dust particles. The Aitken and nucleation modes consist of particles that are emitted directly from combustion sources, or condense from cooled gases soon after emission, and have a short lifetime (less than one hour) because they rapidly coagulate to form larger particles or serve as nuclei for droplets (Cubison et al., 2006).

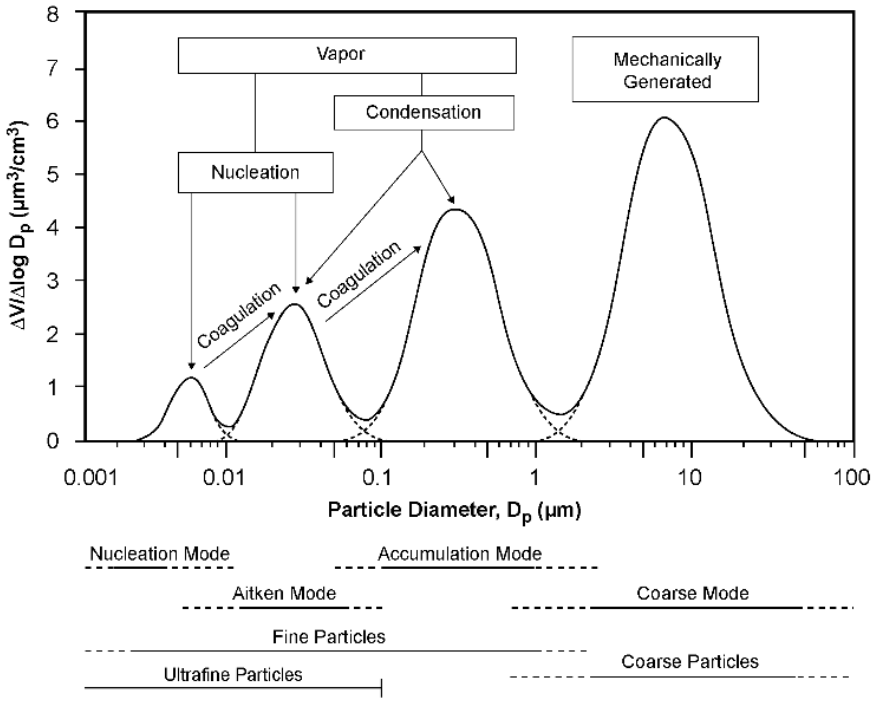

Fig. 1: Idealized modal classification; coarse, accumulation, Aitken and nucleation modes as proposed in EPA's air quality criteria document (2004).

2) The classification method based on the capability of particles to penetrate into various compartments of the respiratory system classifies PM in three groups: inhalable, thoracic, and respirable particles. Inhalable particles enter the respiratory tract and are mostly retained in the head airways; thoracic particles pass the larynx and reach the lung airways and partly the gas-exchange regions of the lung; respirable particles easily reach the gas-exchange region of the lung, the alveoli (EPA, 2004; Wang et al., 2009). Deposition of particles in the lung will be treated more in detail in the chapter 1.2.

3) PM can also be classified accordingly to a size-selective sampling: this method is based on PM samplers efficiency curves and particle size is usually defined by the 50% cut point. Thus PM10 refers to all the particles

collected by a sampling device that collects the 50% of 10 μm particles and rejects the other 50%. Another consequence of this definition is that for PM10, some particles with AED > 10 μm are collected, thus the samples will be not constituted only by particles with AED < 10 μm.

Thus referring to PM10 as particles with aerodynamic diameter < 10 μm, is a simplification that can be used as long as it is made clear that it is not entirely correct.

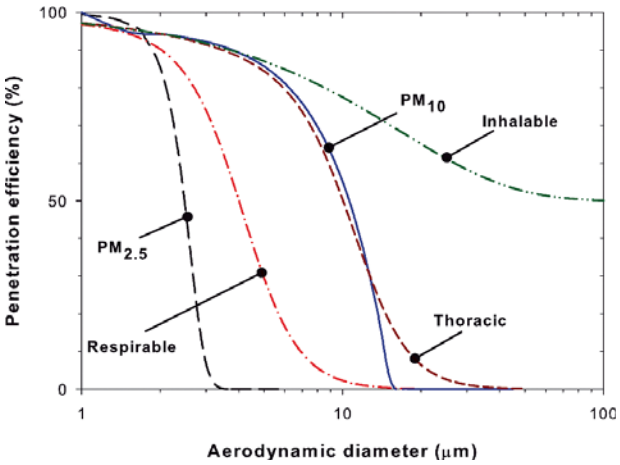

Fig. 2: PM2.5, PM10, inhalable, thoracic, and respirable particulate matter sampling conventions promulgated by US-EPA (Wang et al. 2009)

Moreover, the size-selective sampling method is the one used for definition of air quality standards and legislature and, as a consequence, it is the most widely used in literature, especially in toxicological studies that sample PM in the atmosphere. PM10 and PM2.5 have been selected as standard sizes to a regulatory aim, on the basis of health considerations; PM10 was chosen in order to consider particles small enough to enter the thoracic region of the human respiratory tract, since PM10 sampling efficiency curve matches with the thoracic PM (Fig. 2). The PM2.5 sampler was designed to separate fine and coarse particles on the basis of their different properties (EPA, 2004),

thus PM2.5, or fine PM, comprehends a part of the respirable PM, capable of deeper penetration and higher deposition in airways and lungs (Fig. 2).

Recently more and more attention has been reserved to the ultrafine particles, with an AED < 0.1 μm (UFPs or PM0.1), for the toxic potential related to the small size, high number concentration and large surface area per unit mass of this class of PM; these characteristics determine peculiar behaviours of UFPs as increased adsorption of organic molecules and enhanced ability to penetrate the lung and possibly also systemic circulation (Li et al., 2003).

1.1.2. *Structure and chemical composition*

Since ultrafine/fine and coarse PMs are generated by different processes, they not only differ for size but also for shape, structure and chemical composition (Fig. 3a; Phalen, 2004). PM components can derive from anthropic and natural sources including combustion processes (traffic, warming, waste and industrial incineration, fires), traffic-related abrasion processes (road and car brake debris), dusts resuspension from soil or soil erosion and biological material (Schwarze et al., 2006). Ultrafine and fine PMs are mainly generated by combustion sources and observed under an electron microscopy appear to be composed by aggregates of small round-shaped particles (Fig. 3b) of 20-40 nm. These nanoparticles are formed by an inner core constituted by elemental carbon (EC) or soot, that is generated during the combustion process and functions as a condensation nucleus for a very heterogeneous group of components; inorganic ions, metals and organic compounds, as polycyclic aromatic hydrocarbons (PAHs), that are emitted from traffic and biomass combustions, are thus highly represented in fine particles (Pope and Dockery, 2006).

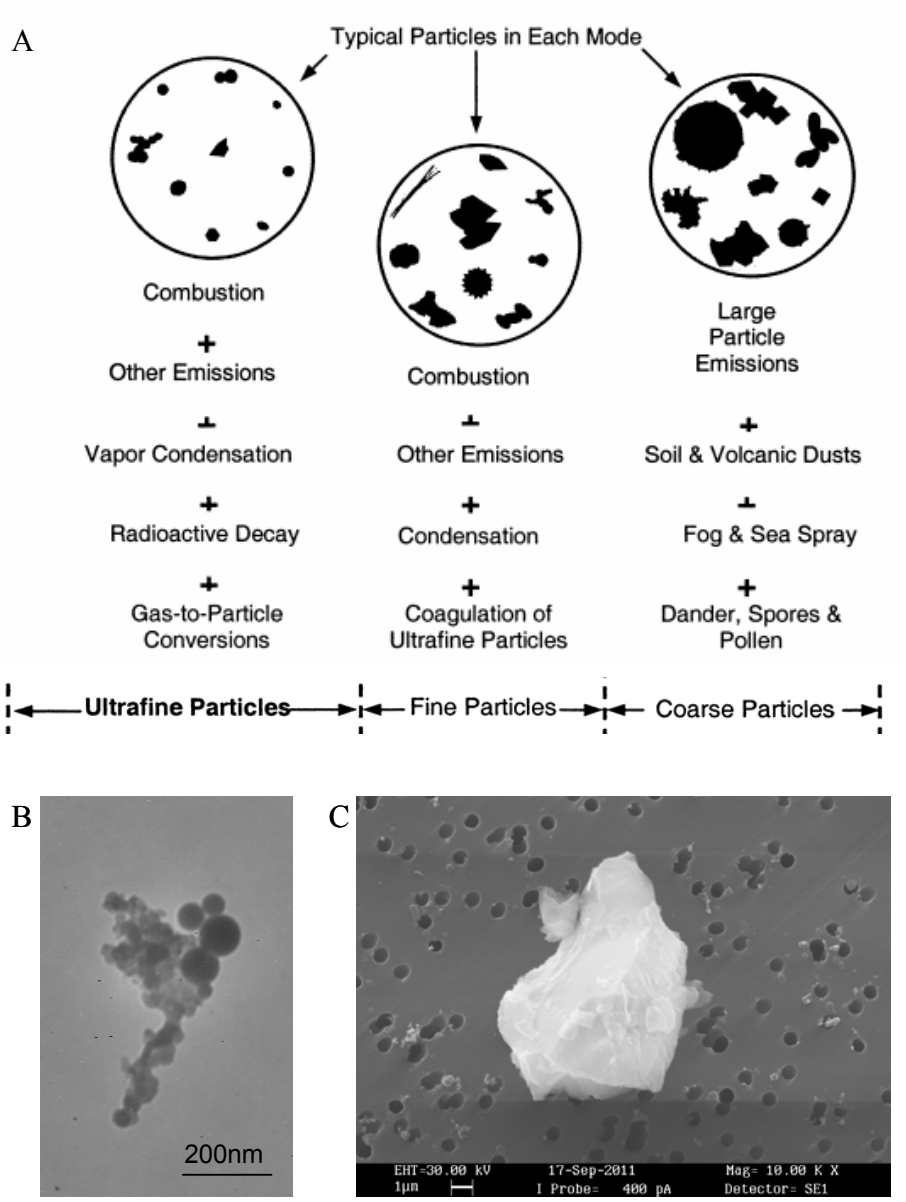

Fig. 3: PM structure. a) Semplified representation of typical shapes and sources of particles in each mode (Adapted from Phalen, 2004). b) TEM image of PM2.5. c) SEM image of a sodium-aluminosilicate particle in PM10.

Coarse PM is mainly generated by mechanical degradation of materials and contains particles of different shapes, regular if derived from crystalline structure or irregular if formed by friction forces among different surfaces (Fig. 3c). The coarse fraction is dominated by crustal elements (calcium, aluminium, silicon, magnesium, iron), metals originated from car brake and tire debris (lead, zinc, nichel, chromium, iron, cadmium, copper) (Adachi and Tainosho, 2004) and biological material as endotoxins, bacteria, fungal spores, and pollen (Pope and Dockery, 2006; Schwarze et al., 2006).

However, the physico-chemical properties of particles not only vary with size but also with time, season and location; in fact differences in meteorology, emission sources and topography contribute to the dispersion, transformation and removal of PM from the atmosphere, resulting in variations of particles concentrations, composition and properties (Schwarze et al., 2006; Kim et al., 2005; Giri et al., 2008). Wind speed and direction determine the range of the transportation of PM from the sources (Mooibroek et al., 2011) and the intensity of soil erosion and resuspension; solar radiation, temperature and humidity induce photochemicals reactions of compounds present in the atmosphere and, besides the chemical modification of the molecules, determine their capability to condense on particles; precipitations largely removes pollutants from the atmosphere (Giri et al., 2008).

Sources may differ in relation to location and season; traffic, heating from biomass, incineration, result in an higher concentration of organic compounds and ultrafine particles, which will be thus representative of sites closed to vehicular and combustion emissions (Sardar et al., 2005); industrial activities are often related to high emissions of metals while sea spray is a main contributor of PM in marine locations (Mooibroek et al., 2011).

Finally, the topographic features can affect PM composition and concentration, acting as barriers preventing the transportation of PM from/to other areas (EPA, 2009). This is the case of Pianura Padana, where the city of Milan is located, a valley closed by muontains on the north and south side where air pollutants are restrained.

1.2. *The respiratory system and particles deposition*

The respiratory system is the primary target of airborne particles which are inhaled and tend to accumulate in the airways. The exposition dose of the tissues depends on the PM atmospheric concentration, its deposition rate in the airways, clearance mechanisms and subsequent retention of particles within the respiratory tract; all these parameters thus determine the particles dosimetry. The comprehension of PM dosimetry is of pivotal importance to understand the toxic potential of this compound. In this respect, the respiratory tract can be divided into three main regions: the extrathoracic or head region, the tracheobronchial region and the alveolar or pulmonary region, where gas exchange occurs (EPA, 2004; Fig. 4a).

The most important PM property driving deposition rate in the airways is particles size: in fact inhaled particles penetrate the respiratory system depending on mechanisms, such as inertial impact, sedimentation, diffusion, electrostatic attraction, which are related to particles size (EPA, 2004). Besides PM dimension, other characteristics of the particles of importance in determining the dosimetry of PM are the particle shape, solubility and chemical composition. Moreover the dosimetry is affected by features typical of each person, such as breathing parameter, morfo-functional condition of the respiratory system and differences in the anatomy of individuals; given the large amount of parameters that must be taken into account to study the deposition of PM, dosimetry models have been

developed to describe the behaviour of particles in the airways, considering the various parameters involved (Phalen, 2004; Asgharian et al., 2003).

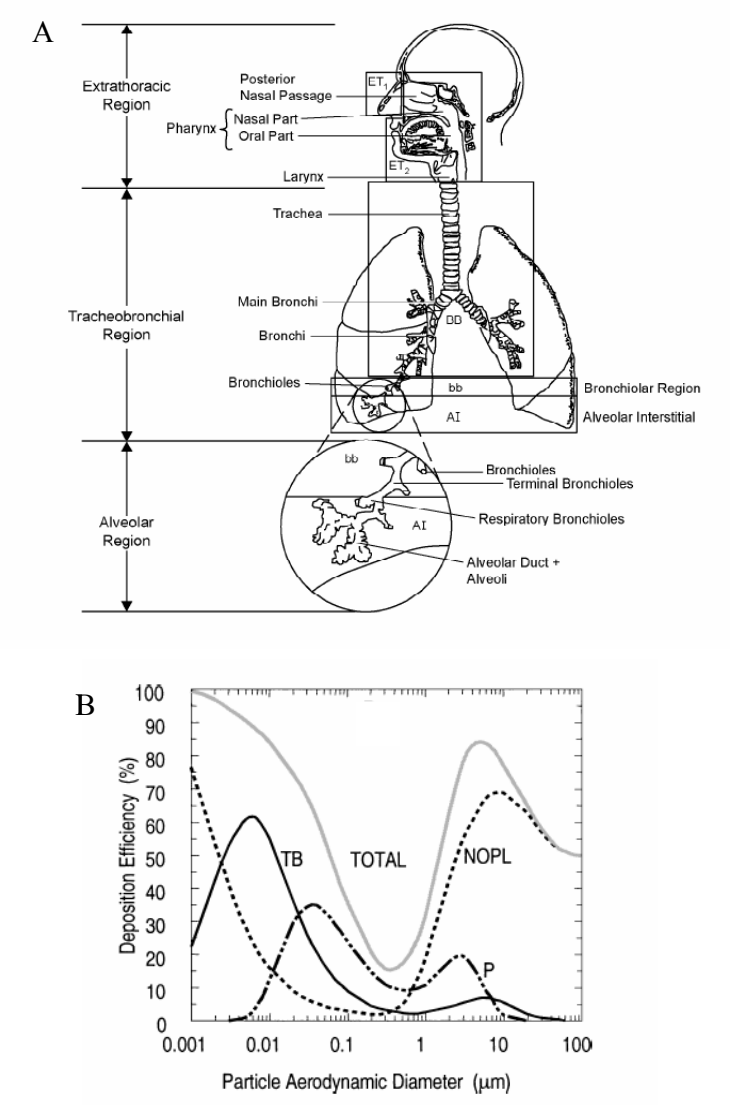

Fig. 4: The respiratory system (a; EPA, 2004) and particle deposition efficiency in the different respiratory regions (b; Phalen, 2004). NOPL: head region (naso-oro-pharyngeal-laryngeal), TB: tracheobronchial region, P: pulmonary region.

In an adult engaged in low-level physical activity, the major part of particles with an AED of 10 μm deposits in the head region, while smaller particles are able to reach deeper regions of the respiratory system (Fig. 4b; Phalen, 2004). Noteworthy, the ultrafine particles not only deposit at tracheobronchial and pulmonary levels but a significant percentage of these particles is restrained in the head region.

However, the distribution of air flow inside the respiratory system is complex and uneven, and as a consequence, particles deposition on the lung surface is not uniform and some sites exhibit higher deposition levels (EPA, 2004). In particular, enhanced deposition has been shown to occur at branching points in the tracheobronchial and alveolar regions and this "hot spots" distribution of particles could be an important factor in eliciting pathophysiological effects.

Each region of the respiratory system presents typical clearence mechanisms that protect the organism from inhaled external agents and particles that deposit in the different tracts encounter such different pathways. Filtration of the nasal passages is the main protective mechanism in the head region, while mucus secretion and ciliated cells act to remove particles and other external bodies in the tracheobronchial region. At the alveolar surface the clearance machinery is constituted by macrophages and type II pneumocytes that perform particles removal through their phagocytic activity. Particular attention should be finally paid to ultrafine particles which, thanks to their tiniest dimensions, may be able to escape macrophages removal and penetrate the alveolar-capillary barrier (Oberdörster, 2001).

1.3. *PM exposure and health effects*

The adverse effects of PM on human health is by now well established (de Kok et al., 2006; Englert, 2004; Schwarze et al., 2006) and can be divided into local effects, that rise in the lung, and systemic effects, that impact on

the cardiovascular system (Donaldson and MacNee, 2001). Numerous epidemiological studies, based on hospital and emergency department admissions, have reported consistent associations between PM atmospheric concentrations and onset/exacerbation of respiratory and cardiovascular diseases as well as mortality in the population. Although all population is affected, susceptibility may vary with health state, age and inter-individual variability in exposure and response.

Effects have been related both to short-term and long-term exposures to PM; short-term exposures have been reported to induce exacerbation of airways disease in patients with asthma and Chronic Obstructive Pulmonary Disease (COPD) (Donaldson et al., 2003; Katsouyanni et al., 2001), onset of cardiovascular diseases (EPA, 2009) and increased mortality for heart attacks and respiratory causes (Donaldson and MacNee, 2001). The importance of particles size on these outcomes has been evaluated and positive relations both with PM10 and PM2.5 were found (Samet et al., 2000; Schwartz and Neas, 2000); the coarse fraction (PM10-2.5) resulted to be as strong as fine PM in increasing hospital admissions for respiratory diseases (Brunekreef and Forsberg, 2005), while the correlation with cardiovascular diseases is weaker (Schwarze et al., 2006). Studies conducted in European and United States cities reported short-term exposure to PM10 to increase mortality of a value ranging from 0.46 to 0.62 % (mean 0.5 %) for each 10 $\mu g/m^3$ increment in the daily concentration (Cohen et al., 2004; Katsouyanni et al., 2001). Therefore a PM10 concentration of 100 $\mu g/m^3$ is expected to increase daily mortality of about 5 %.

Long-term effects are sneakier and can occur at lower concentrations. Long-term exposure epidemiological studies showed associations with PM2.5 but not with larger size fractions; fine PM has been linked with increases of risk of cardiovascular mortality (Dockery et al., 1993; Pope and Dockery, 2006; Brook et al., 2010; Pope et al., 2011) and of lung cancer (Turner et al., 2011;

Pope et al., 2002; Pope et al., 2011). These associations have been found on the basis of American Cancer Society (ACS) and of the Harvard Six-Cities data (Dockery et al., 1993; Pope et al., 2002) comparing with the historical mean PM2.5 concentration of 20 μg/m^3 (9.0–33.5 μg/m^3) and 8 μg/m^3 (11.0–29.6 μg/m^3) respectively.

Thus, health effects elicited by PM occur at concentrations currently found in many cities and the risk for various outcomes has been shown to increase with exposure levels; accordingly to this observation, both daily and annual average concentration threshold for PM2.5 and PM10 have been indicated by the WHO (2005) to protect the health of the population. Guideline values have been chosen on the basis of evidences reported in literature: for PM10, the short-term guideline (24 hour average) is 50 μg/m^3 while the value for PM2.5 is 25 μg/m^3, derived from the corresponding PM10 value by application of a PM2.5/PM10 ratio of 0.5, typical of developing country urban areas. The annual average concentration chosen as the long-term guideline value for PM2.5 is 10 μg/m^3 (WHO, 2005), representing the lower end of the range over which significant effects on survival were observed in the ACS study (Pope et al., 2002). Accordingly, the annual average concentration chosen for PM10 was 20 μg/m^3 even if the quantitative evidence on coarse PM is considered insufficient. Actually, till now it was not possible to suggest a threshold below which no adverse health effects occur: the lower concentration at which adverse health effects has been demonstrated is not greatly above the background concentration, which for PM2.5 has been valued to be 3-5 μg/m^3 (WHO, 2005). Taking in account these informations, and considering individual susceptibility, it is unlikely that any air quality improvement strategy that only consider atmosphere concentration of PM will lead to a complete protection of population against adverse health effects of PM.

In fact, not only exposure levels are responsible of biological effects elicited by PM but different particle properties are involved (Schwarze et al., 2006). To discover which PM fractions or constituents are more critically involved in eliciting adverse health effects is thus crucial the implementation of strategies to improve air quality based on toxicological analysis.

1.4. *PM toxicity: mechanisms and outcomes*

The difficulty of investigating PM toxicity partly lies in the variability and complexity of this pollutant, whose heterogeneity in structure and composition can lead to different biological outcomes. In order to understand the epidemiological findings, the toxicological studies have an important role providing controlled and simplified models that can help to clarify the mechanisms of PM toxicity and the molecular pathways involved. Moreover, comparative analyses of different PM classes may help to associate a specific biological effect to a particular physico-chemical characteristic and thus correlate the outcome with particles properties.

In vitro models are of fundamental importance in understanding the molecular pathways activated by PM exposure at cellular level, which in turn might explain the biological outcomes observed in humans. The models used for PM toxicity analysis include various alveolar and bronchial epithelial cell lines and monocyte/macrophage cells; endothelial cell lines and co-coltured systems of these models have been used to investigate possible cardiovascular effects (Wang et al., 2010; Qu et al., 2010). However, due to the intrinsic limitation of the *in vitro* models, *in vivo* systems have been widely used to investigate and clarify the systemic outcomes related to PM exposure.

1.4.1. *Inflammation*

Airways inflammation has been found to be a crucial short-term effect of PM exposure in humans (Jalava et al., 2008; Hetland et al., 2005; Dye et al.,

2001). Inflammation is a central response of the organism against adverse stimuli but, when it occurs with a high intensity or for a long period, it is at the base of airways inflammation and lung diseases such as COPD and asthma. This process is characterized by the release of pro-inflammatory mediators (cytokines, chemokines, reactive oxygen species, etc.) by alveolar macrophages (AM) and pulmonary epithelial cells. The cytokines are signalling proteins involved in regulation of many physiological events; in the inflammatory process they can act as pro- or anti-inflammatory mediators. Chemoattractants cytokines (chemokines) are produced and released in case of injury or various stimuli, with the aim of recruit and activate the immune cells (Ramgolam et al., 2008). Interleukins 8 and 6 (IL-8 and IL-6) are well known and widely studied pro-inflammatory cytokines; despite some functional redundancy, these proteins also present specific functions. IL-8 is the first line of cellular defence since is fast released after the stimuli and immediately elicits the recruitment of immune cells, in particular neutrophils, basophils and macrophages, at the site of infection; its effect is rapid and long lasting since IL-8 persists in active form after being released (Kocbach et al., 2008; Baggiolini and Clark-Lewis, 1992). IL-6 is involved in the regulation of the acute-phase response to injury and inflammation and induces the final maturation of B and T cells into antibody-producing ce1ls (Kishimoto et al., 1995). The regulation of these cytokines is performed by a complex molecular pathway that involves the activation of other mediators including tumor necrosis factor (TNF) and interleukin-1β (IL-lβ) (Eskan et al., 2008). IL-1β is a pro-inflammatory cytokine involved in many cellular functions, such as proliferation, activation of the immune response through the induction of other interleukines (such as IL-6 and IL-8) and, noteworthy, it has a critical role in the systemic phase of inflammation (McColl et al., 2007).

Local inflammation may result in a systemic response that can subsequently contribute to cardiovascular effects and increase the risk of stroke (McColl et al., 2007). The explanation of such effect in relation to PM exposure is that ultrafine particles, soluble PM components or inflammatory mediators may translocate from the lung to the circulatory system and exert there their action (Schwarze et al., 2006; Mills et al., 2007); in fact it has been demonstrated that alveolar macrophage-derived IL-6 mediated pro-coagulation effects in mice exposed to PM10 (Mutlu et al., 2007), providing a link between lung cytokines and systemic responses. In this respect IL-1β may be a major contributor to the systemic responses induced by PM inhalation.

Many *in vitro* and *in vivo* studies have reported the release of IL-6 and IL-8 in cell lines exposed to various PMs (Becker et al., 2005; Hetland et al., 2000; Mutlu et al., 2007; Jalava et al., 2008). Inflammatory potential of PM has been mainly related to coarse particles, while fine and ultrafine PMs have been found to be less effective (Monn and Becker, 1999; Hetland et al., 2005; Schins et al., 2004). This observation has been linked to a higher endotoxin content in the coarse fraction (Schins et al., 2004; Hetland et al., 2005; Kocbach et al., 2008); endotoxins, such as lipopolysaccharides (LPS), are components of Gram negative bacteria wall, which are known to induce inflammation in cells through the activation of specific Toll-like receptors (TLRs). The activation of these receptors is one of the steps required for IL-1β release, since it leads to the production of the IL-1β precursor (pro-IL-1β); however the maturation and subsequent release of this cytokine entails the cleavage by caspase-1 of pro-IL-1β to IL-1β (Eskan et al., 2008). Actually many studies on PM effects suggest that endotoxin is not the sole factor eliciting PM-induced inflammation; metals contained in particles have been related to the inflammatory potential of PM (Becker et al., 2005; Hetland et al., 2000; Øvrevik et al., 2005; Veranth et al., 2006). The metals-

related inflammatory effect has been partly ascribed to their capability to induce formation of reactive oxygen species (ROS) and thus oxidative stress (Fig. 5; Donaldson and MacNee, 2001).

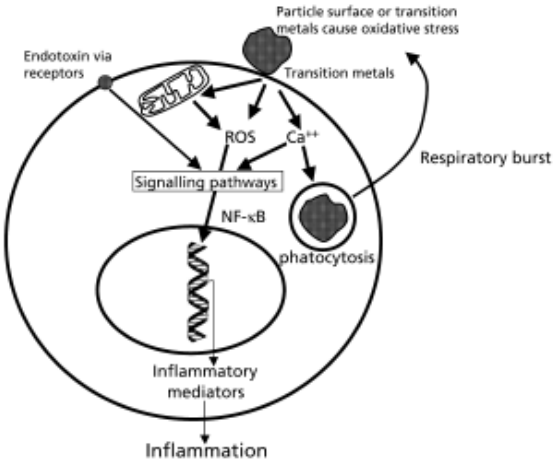

Fig. 5: Hypothesis of inflammation mechanism in cells exposed to PM (Donaldson and MacNee, 2001).

These studies demonstrated that PM inflammatory potential depends on chemical composition of particles rather than the dimension per se. The coarse fraction is a potent inducer of inflammation due to the presence of specific components which are not present, or minimally, in the fine PM. The relation "PM composition – source – dimension" is thus a parameter that may be far more important for the comprehension of PM effects rather than simply the size of the particles (Schwarze et al., 2006).

1.4.2. *Genotoxicity*

Toxicological studies widely described the genotoxic effect of PM in humans (Møller et al., 2008; Billet et al., 2008; Oh et al., 2011). These results, together with the epidemiological evidences of lung cancer mortality for long-term exposure to PM2.5, suggest a role for DNA damage in PM-related health effects (EPA, 2009).

In agreement with epidemiology, fine PMs usually have a higher genotoxic potential compared to coarse fractions, in *in vitro* and *in vivo* models (Chackra et al., 2007; de Kok et al., 2005; Park et al., 2005); moreover, PMs from industrialized, traffic and wood/biomass combustion areas have been found to exert a stronger effect on DNA (Shi et al., 2006; EPA, 2004). The high genotoxicity of these PMs is due to the particles composition and source, since particles derived from combustion processes have been found to be rich in metals and organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), which are notorious genotoxic elements (Mehta et al., 2008).

Actually, different mechanisms have been reported for PM-induced DNA damage, which include oxidative stress, organics metabolites formation and direct interaction of particles organic compounds with the DNA (Fig. 6; Møller et al., 2008). Generation of ROS and consequent oxidative stress is widely recognized as an important toxicological mechanism in PM induced DNA double strand breaks/oxidation and possibly lung cancer (Shi et al., 2006; Møller et al., 2008); PM-induced ROS are formed through numerous mechanisms including Fenton reaction of soluble metals, metabolism of organic compounds as PAHs, quinones redox cycling, cell membrane interactions caused by physicochemical surface properties of particles, NADPH-oxidase activation, secondary effects on mitochondrial damage and response to phagocytosis (Knaapen et al., 2002; Li et al., 2008). Besides ROS formation, the metabolic activation of PAHs by P450 cytochromes (CYP1A1/1B1) forms reactive species, epoxides, that may form bulky adducts on the DNA (Billet et al., 2008). The formation of such kind of adducts is a well known process in cells and animals exposed to PAHs, such as benzo[a]pyrene (BaP) (Park et al., 2009; Jiang et al., 2006). BaP is indeed one of the organic molecules present in fine PMs and it is thus likely that the organic fraction released from particles could activate such processes.

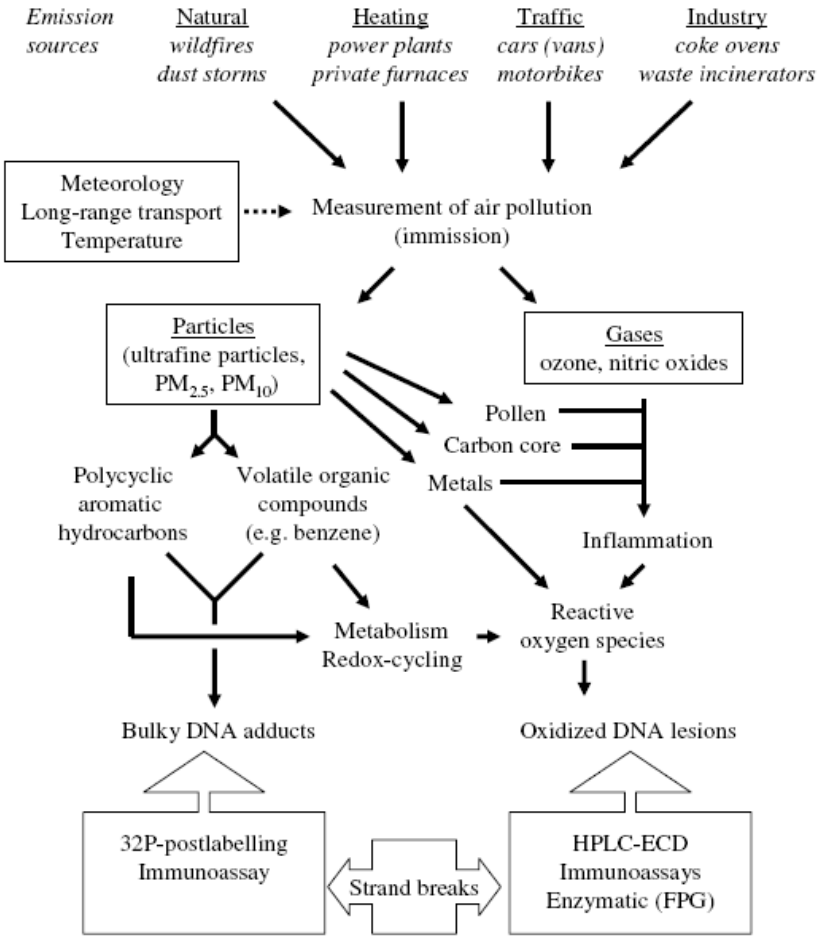

Fig. 6: Mechanisms of PM-related DNA damage induction (Møller et al., 2008)

Interestingly, it has been recently demonstrated that transition metals (Cr, Cd, Ni and arsenic) and aldehydes present in PM can inhibit DNA damage repair mechanism and this effect may enhance the genotoxic effect of PM and contribute to lung carcinogenesis (Mehta et al., 2008). In fact, when DNA damage is present, the cell activate different repair mechanisms whose initial step is the DNA lesions recognition and binding by specific sensors as

ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related); these proteins recognize quite different kind of damage, ATM being more specific for double strand breaks (DSBs) and ATR for single strand breaks (SSBs) and stalled replication forks (Paulsen et al., 2007; Yang et al., 2004). Both the sensors phosphorylate the histone 2AX, a protein which is present in the nucleosome and induces chromatin changes around the lesion site, leading to the recruitment of many checkpoint and repair factors (Dickey et al., 2009; Branzei and Foiani, 2008). However upon DNA DSBs formation, phosphorylated H2AX (γH2AX) appears in chromatin as discrete foci, with each focus representing a single point of DSB (Marti et al., 2006; Rogakou et al., 1999). Thus the presence of γH2AX foci has been adopted as clear and consistent quantitative marker for DSBs (Dickey et al., 2009; Albino et al., 2009), the most dangerous DNA lesion that can result in genetic abnormalities leading to cancers.

The DNA damage detection is then followed by the activation of transcription factors which induce the expressions of genes involved in DNA repair, cell cycle arrest and, eventually, cell death (Yang et al., 2004); in fact, in presence of DNA damage, DNA-integrity checkpoints function to delay the cell cycle progression providing time to repair the lesions before entry in the subsequent phase of the cell cycle (Fig. 7; Pearce and Humphrey, 2001). The main checkpoints are at G1/S and G2/M transitions and among the transcription factors involved in these pathways the more important are the tumor suppressor p53, the Chk1/Chk2 kinases and the complexes cyclin-dependent kinases (CDKs)-cyclins.

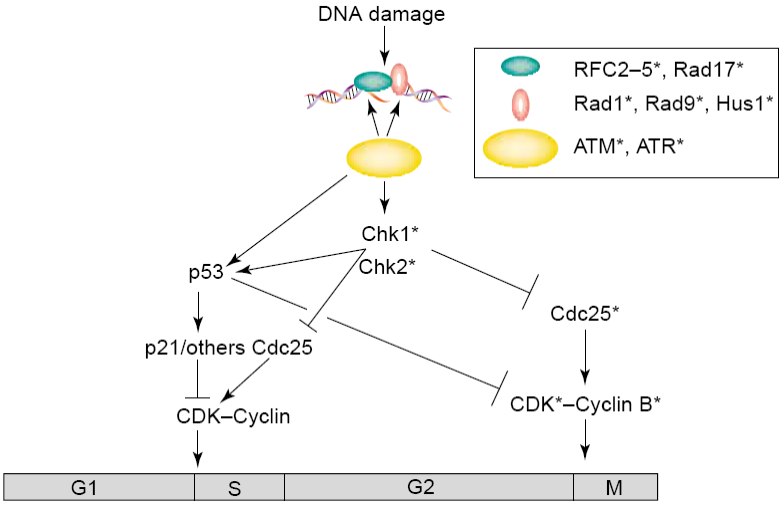

Fig. 7: Cell cycle regulation through DNA-integrity checkpoint pathways (Pearce and Humphrey, 2001).

A further checkpoint, located inside mitosis at the metaphase/anaphase (M/A) transition, is the spindle assembly checkpoint (SAC) that functions to delay mitosis until the mitotic spindle is correctly formed. The activation of SAC can be related both to the presence of DNA damage and to other kinds of damages, e.g. structural damages in presence of spindle poisons that prohibit the stable attachment of all kinetochores or in case of an incorrect attachment of the spindle to the kinetochores (Rieder, 2011). Mitotic arrest at the M/A transition and aberrations of the mitotic spindle have been reported in V79 cells (chinese hamster fibroblasts) exposed to PM10 organic extract (Glowala et al., 2001; Motykiewicz et al., 1991); these results likely indicate the involvement of SAC, however the mechanisms lying behind this effect have never been investigated.

Finally, if the cells are not able to repair the damage and satisfy the checkpoints within a certain period of time, other mechanisms can be triggered to protect the organism; the most important of these mechanisms are active programs of cell death (Yang et al., 2004).

1.4.3. *Cell death*

PM-induced damages as high to prevent the recovery through repair mechanisms may exert a cytotoxic effect leading to cell death. Death can occur in cells roughly by two distinct mechanisms: one, necrosis, caused by factors external to the cells, the other, apoptosis or generally programmed cell death (PCD), depending on the activation of death pathways within the cells. The specific mechanisms triggered by PM depend on the chemical properties of the different fractions but also on the exposure dose (Nel et al., 2001); in fact the same stimuli which induce apoptosis at low doses can result in necrosis at higher doses (Elmore, 2007).

Necrosis is a passive death process characterized by the failure of the cell to maintain homeostasis, by subsequent cell lysis and release of the cytoplasmic contents into the extracellular space (Fink and Cookson, 2005). This situation, *in vivo*, contribute to the inflammation and the development of both acute health effects and chronic lung diseases; in fact, the release of inflammatory mediators from the damaged epithelium, sustains and prolongs the inflammatory reaction and possibly leads to chronic effects (Schwarze et al., 2006).

Apoptosis is a form of PCD caspase-mediated and characterized by specific biochemical and morphological features (Elmore, 2007; Fink and Cookson, 2005). The caspases related to apoptosis are protease with specific targets and can be divided in initiators of the process, as caspase-2, -8 or -9, and effectors, as caspase-3, -6 or -7 (Fink and Cookson, 2005). The activation of effector caspases produces the cleavage of DNA and cytoskeleton proteins that leads to the typical morphological features of apoptosis: nuclear fragmentation, cell shrinkage, membrane blebbing, chromatin condensation, formation of apoptotic bodies with intact membrane and exposure of

phosphatidylserine, molecules targeting cell corpses for phagocytosis. Two main apoptotic pathways have been identified:

i) the extrinsic or death receptor pathway is TNF-α-related, since high levels of this protein provoke caspase-8 and -3 activation (Elmore, 2007). This pathway is partly related to the inflammatory process and ROS formation since both of these events induce high levels of TNF-α;

ii) the intrinsic or mitochondrial pathway involves the release into the cytosol of cytochrome c, a protein normally confined in the mitochondrial intermembrane space; cytochrome c in the cytosol converts the pro-caspase-9 to the active form which promotes apoptosis through caspase-3 activation (Elmore, 2007). This mechanism involves a complex of proteins including the tumor suppressor p53, which regulates the transcription of many genes and the activity of pro- and anti-apoptotic proteins of the Bcl-2 family, which govern mitochondrial membrane permeability. This pathway is related to the presence of DNA damage, which provokes the activation of the pro-apoptotic proteins, responsible of cytochrome c release (Dagher, 2006). However, also high levels of ROS may activate the apoptotic mitochondrial pathway, directly causing mitochondrial damage (Upadhyay et al., 2003).

A particular feature of apoptosis is mitotic catastrophe, a type of cell death that occurs during mitosis in presence of DNA and/or cellular damage (Castedo et al., 2004). Cell death occurs during the M/A transition and is characterized by the activation of caspase-2, in response to DNA damage, and/or mitochondrial membrane permeabilization with the release of cytochrome c and subsequent activation of the caspase-9 and -3.

In cells exposed to PM both necrotic and apoptotic processes have been widely reported (de Kok et al., 2006; Schwarze et al., 2006); necrosis is often related to a severe inflammatory status or oxidative stress characterized by high levels of released cytokines, ROS formation and the presence of

various metabolites which contribute to epithelial damage (Schins et al., 2002). Intrinsic and extrinsic apoptotic pathways have been described especially for PM2.5; this fraction has been found to induce apoptosis through the TNF-α-dependent pathway and by the mitochondrial pathway. Both pathways have been related to severe DNA damage and ROS formation (Fig. 8; Dagher, 2006; Upadhyay et al., 2003; Sánchez-Pérez et al., 2009).

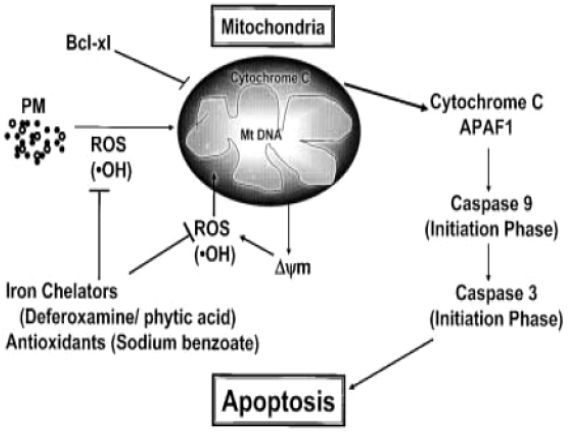

Fig. 8: Hypothetical model of apoptotic mitochondrial pathway triggered by PM through ROS formation (Upadhyay et al., 2003).

PM-induced apoptosis and necrosis have also been reported in primary human AM, and the onset of the apoptotic process was related to the organic components of PM (Obot et al., 2002); noteworthy, murine AM have been found to behave in a similar manner (Obot et al., 2004). Finally, i*n vivo* studies confirmed the findings obtained in cell lines reporting the presence of ROS-related apoptotic processes in mice exposed to PM2.5 (Laing et al., 2010).

However, as previously stated, the kind of death process initiated, necrosis or apoptosis, can be determined also by the dose of exposure in cell culture;

for instance, the organic compounds present in diesel exhaust particles (DEPs) are known that induce apoptosis in cells through the mitochondrial pathway (Yun et al., 2009), though higher particle doses have been reported to induce necrotic events (Nel et al., 2001).

1.5. *Perspectives*

The comprehension of the biological effects induced by airborne PM is a pivotal issue for the risk that inhalation of this compound poses for human health. Despite the large amount of data present in literature, there are still unresolved questions regarding 1) the cellular mechanisms involved in the induction of the diverse adverse effects and 2) the PM components which determine the biological responses.

Knowing the cellular pathways activated in response to PM exposure is a key step for to the understanding of the clinical outcomes, while the identification of the more hazardous components may help to define more efficient strategies to reduce air pollution by focusing on those sources which emit the most armful particles and to refine the guidelines for air quality improvement.

The PM complexity makes such kind of investigation particularly difficult since the variability in structure and composition leads to a large variety of biological effects (cytotoxicity, inflammatory effects and genotoxicity) when comparing PMs from different cities or seasons. In this perspective, a comparative analysis of PM classes, sampled in different seasons and locations, may help to associate specific biological effect to particular physico-chemical characteristics. Furthermore *in vitro* systems, although some limitations are useful models to investigate the molecular pathways involved in PM toxic effects.

2. Aims of the thesis

The main objective of this thesis has been to examine the biological effects and mechanisms induced by PM exposure in representative lung epithelial cell lines, linking these effects to specific particle properties, such as the dimension, the chemical composition and the biological components eventually present.
In order to fulfil this purpose the following specific aims were pursued:

- Evaluation of the role of season and location, on the PM toxic potential by sampling the particles in different periods of the year and sites.

- Determination of the role of size of particles with regard to specific biological effects of the different PM classes.

- Exploration of possible mechanisms and molecular pathways activated by Milan winter PM2.5 exposure linking the outcomes to particle physical and chemical properties.

3. Summary of findings

3.1. *PM effects: season- and location- related variability*

The first phase of the research regarded the season- and place-related variability of the effects triggered by PM1 and PM2.5; PMs were sampled during spring, summer, autumn and winter 2008-2009, in three Lombardia sites characterized by diverse human impacts. The urban site (Milan, Torre Sarca) has been chosen as representative of a traffic area with main emissions from vehicle combustion sources; the rural site (Oasi Bine, Mantova) is a protected area (WWF) characterized by agricultural activities and related sources; the remote site (Alpe San Colombano, Sondrio) is located at 2280 m above sea level in the alpine area and, because of its position, is far from human activities; the only sources of PMs are thus related to atmospheric transport. The analyses, part of the VESPA project, focused on the effects on viability, pro-inflammatory response and ROS formation in A549 cells exposed for different time points to 6 μg/cm^2 of the different PMs. The chemical characterization of the particles was performed in collaboration with the group of Atmospheric Chemistry of the University of Milano-Bicocca, Department of Environmental Sciences (DISAT) partner of the project.

The results of the toxicological analyses, reported in Supplementary data section I, showed that the biological outcomes were related to the site and season of sampling. The cell viability, measured at 24 h by MTT assay (that determines the metabolic activity of cells) and LDH release (an index of plasma membrane integrity), showed that the winter remote site's PMs were highly cytotoxic, while summer urban and rural PMs reduced the metabolic activity at a lower extent. The inflammatory response was induced only by winter PM1 sampled in the remote site, while ROS formation was enhanced by summer and autumn urban and rural sites' PMs.

These results underlined the role of season- and location-related characteristics of PMs in determining different biological effects. Moreover slight differences have been observed between the fine PMs sampled in the same area. Accordingly to the results obtained, the further research focused only on Milan PM to better discriminate the role of season- and size-related characteristics. In order to fulfil this aim the number of seasons investigated has been reduced, selecting summer and winter, while the number of PM fractions has been increased.

3.2. *PM effects: season- and size-related variability*

The second part of the thesis regarded the effects of PM0.4, PM1, PM2.5 and PM10 sampled in Milan during summer and winter 2009-2010. In this study, which was a part of TOSCA project (supported by CARIPLO foundation), quasi-ultrafine (PM0.4) and PM10 have been also considered to analyze a wider range of particles sizes. The endotoxin content of particles and their shape and dimension have been examined to better specify the physico-chemical properties of PMs. Besides, THP-1 monocytes have been used in mono-culture and in co-culture with A549 cells to obtain an *in vitro* system more representative of the cellular interactions (between epithelial cells and monocytes/macrophages) that normally happen *in vivo* at the alveolar space. Cytotoxicity, inflammatory response and ROS formation have been analysed in cells exposed to 10 $\mu g/cm^2$ of PMs. Other toxicity markers have been considered in this part of the research: genotoxicity, cell cycle alterations and expression of specific proteins related to the biological effects observed, such as cytochrome P450 CYP1B1 and γH2AX, were useful to investigate the possible mechanisms of action of PM.

The quasi-ultrafine PM0.4 has been analysed to investigate the toxic effects related to the nanometric dimension and high surface/volume ratio of these particles. Somehow surprisingly, any typical behaviour has been associated

to this fraction, as the biological responses elicited were similar to those of the fine PM1 (Paper I). Accordingly, the morphological characterization showed that PM0.4 is similar to PM1 in terms of shape and dimension, these fractions being mainly represented by aggregates of 20-160 nm, thus suggesting that PM0.4 and PM1 may be considered together as representative of sub-micrometric particles (Paper I). Moreover a qualitative analysis showed that the quasi-ultrafine fraction tends to rapidly agglomerate in the cell culture medium, thus increasing considerably the dimension of the particles and likely masking possible specific effects of the nanometric particles.

On the contrary, substantial differences in the effects elicited by summer PM10 and the winter PMs have been reported, underlining the importance of season and chemical composition of particles, partly associated to a size-related effect. Summer PM10 affected the viability, assessed by LDH test after 24 h of treatment, of THP-1 cells and co-cultured cells, while any effect could be observed on A549 cells (Paper I and II). Summer PM10 also triggered the highest pro-inflammatory response, measured as cytokines IL-6, IL-8 and IL-1β release, in THP-1 cells and in A549/THP-1 co-culture system (Paper I). Once again, A549 cell line was the less responsive, showing only a slight release of IL-8 (Paper I and II). The cytotoxic and pro-inflammatory potential of summer PM10 correlated with a significantly higher endotoxin and metals content in comparison with all the other PMs (Paper I and II).

Winter PMs showed a completely different kind of toxicity, consisting in the induction of genotoxic effect. Similar outcomes have been reported for all winter sizes, although fine PMs (especially PM2.5) induced slightly higher responses. ROS formation and DNA damage induction, assessed by the expression of the histone 2AX phosphorylated (γH2AX), have been shown after winter PMs exposure in both A549 and THP-1 cells (Paper I). Cell

cycle alteration, represented by an increase of G2/M cells, occurred in THP-1 but not in A549 cells (Paper II). Finally, overexpression of cytochrome P450 CYP1B1 has been described in A549 cells after winter PM2.5 exposure (Paper II). The DNA damaging effect induced by winter PMs can be related to the higher PAHs content reported for the winter samples when compared to the summer ones (Paper II).

3.3. *Winter PM2.5 effects: mechanisms and chemical components involved*

The third part of this research focused on the winter PM2.5 mechanisms of action, with particular interest on the capability to induce ROS formation, DNA damage and cell cycle alteration as previously observed. Moreover, the role of the organic compounds and of their metabolic activation has been investigated. PM activation of pro-carcinogenic gene pathways has been verified analysing the gene expression modulation in A549 cells exposed to winter and summer PM2.5, and the results have been compared with the genotoxic potential of particles (Paper III). The mechanisms and the causes underlying the cell cycle alteration previously observed in THP-1 cells was more deeply characterized in this cell line (Supplementary data II) and especially in the bronchial epithelial transformed cells, BEAS-2B (Paper IV).

The genome wide screening technique (Affimetrix platform), applied to A549 cells, showed that winter PM2.5 modulated genes implicated in transcription regulation and cancer development (CYP1A1, CYP1B1, TIPARP, ALDH1A3, AHRR, GREM1), accordingly to its higher content of PAHs and its capability to induce DNA damage and ROS formation (Paper III). Thus organic components adsorbed to fine winter PM are likely the determinants of the biological effects observed, when bio-available. Summer PM2.5, used in comparison, modulated only a part of these genes and at a lesser extent.

A further analysis of the cell cycle alteration reported for THP-1 cells (Paper II), showed that the G2/M increase consists of cells arrested or delayed in mitosis with altered mitotic spindle organization (Supplementary data II). However, THP-1 cells are non adherent cells and the analysis of mitotic spindle resulted quite difficult. On the contrary, BEAS-2B, being adherent cells, resulted easier to manipulate and more sensitive to the action of PMs. Results obtained on BEAS-2B cells confirmed and implemented the data on cell cycle arrest: briefly, exposing the cells to winter PM2.5 from 1 to 40 h, a marked increase of G2/M cells occurs already after 3 h of treatment and is maintained up to 24 h. After 40 h, however, G2/M cells in PM-treated samples decreased to control values and at the same time a significant increase of apoptotic cells was reported. Fluorescence microscopy analysis showed that after 3 h the amount of mitotic cells was similar in PM-treated and control samples, thus probably representing cells blocked in G2 due to the G2/M transition check point. However, a significant increase of mitotic cells was observed at 10 h in exposed samples. At 24 h cells in mitosis decreased again, but the number of dead cells was markedly increased.

A further characterization of cells arrested in mitosis at 10 h of exposure evidenced that these cells were prevalently in pre-anaphase (prophase and metaphase) thus they seemed to be maintained in metaphase (or prevented to enter in anaphase) by the M/A transition checkpoint (SAC). Furthermore, aberrations of the mitotic spindle (tripolar, multipolar and incomplete spindles) were also observed and cells stained for γ-tubulin evidenced the presence of centrosomes amplification associated to multipolar spindles.

Expression of cyclin B1, that drives the progression of cells from G2 to mitotic phase, resulted altered in cells exposed to winter PM2.5 for 10 and 24 h. In addition, fluorescence microscopy analysis revealed the presence of cells with large abnormal nuclei in samples exposed to PM2.5 for 24 h;

furthermore, cells with micronuclei (MN) were detected in PM-treated samples and some double-nucleated cells were also observed.

The investigation regarding the possible causes of these effects seemed to indicate ROS-mediated DNA damage as a key event of the process: indeed ROS formation was significantly enhanced 2 h after PM treatment and DNA damage was observed in cells already after 3 h from PM exposure, possibly explaining the G2 increase reported at this time point. Moreover ROS related DNA damage, and consequent G2 phase cell cycle arrest, were partly related to the activation of cytochrome P450 enzymes, since the formation of cyp-linked metabolites of PAHs resulted of importance. Indeed, the pre-treatment of cells with antioxidants/nucleophiles (NAC and Thio) and the CYP enzymes/AhR inhibitor α-naphtoflavone (α-NF) almost completely reduced the DNA damage and G2 increase in PM2.5 exposed cells.

Thus in order to elucidate the importance of the organic compounds in triggering the effects observed for winter PMs, the organic fraction of PM2.5 has been extracted from particles. Both the extracted fraction and the washed particles were tested for ROS formation and cell cycle alteration. The organic fraction revealed to be a more potent inducer of ROS compared to whole PM, while washed particles were almost ineffective. Also the effect on G2/M accumulation at 3 and 10 h was enhanced after exposure to organic fraction compared to whole PM, while washed PM was completely ineffective. After 24 h exposure, however, the whole PM2.5 had still a slight G2/M increase, while the organic fraction of PM2.5 at this time had a slight increase in the G1-phase. Surprisingly, similar effects were observed with the whole PM1 and its organic fraction (Supplementary data III). These differences at 24 h of exposure will be subject of further studies, but somehow suggest a deferred release of the organic compounds from the whole PMs..

4. Discussion and conclusions

The data here presented provide a focus on the biological effects of various PMs and a further characterization of the effects of winter PM2.5 and its mechanisms of action.

The comparison of the effects with the chemical and physical properties of particles allowed determining some of the features that are central in eliciting the toxicity of PM. The PM physico-chemical properties are known to vary in relation with different variables; the kind of sources of emission (both natural and anthropic) is the main parameter that establish the characteristics of particles of various size, area and season (Schwarze et al., 2006), and the data reported in Supplementary data section I clearly showed that the atmospheric transports can be considered a peculiar kind of source. Season-related transformations of chemicals in the atmosphere are another important parameter to be considered: during summer the photochemical reactions, associated to the elevated solar radiation, can modify the PM chemical compounds, while in winter season the low temperatures facilitate the condensation and absorption of the volatile compounds on particles surface (Perrone et al., 2010). The size-ratio contribution, i.e. the percentage of PM2.5 in PM10, or PM1 in PM2.5 etc., is another parameter that is particularly driven by season: in fact during winter the combustion processes represent a more important PM source than in summer, and thus fine and ultrafine particles prevail on the coarse fraction. Indeed in Milan, PM2.5/PM10 ratio is up to 0.80 during winter and 0.60 in summer (TOSCA project final report 2009-2011). All these parameters have to be taken into account analyzing the biological effects of PM and this complexity is the reason for the difficulties in solving the question of PM toxicity.

In this work the chemical composition of particles has been considered as the main feature explaining PM biological effects. However other characteristics as bioavailability of chemical compounds, particles surface properties and reactivity have been shown to be important contributors to toxic potential of PMs (Zerbi et al., 2008).

In the first part of the research, the effect of sampling site of fine PM on the biological outcomes has been investigated and the results, reported in Supplementary data section I, evidenced a different toxic potential for PMs from diverse locations. The main distinction can be done between the remote site (Alpe San Colombano) and the sites situated in the valley: surprisingly, the winter PM sampled in Alpe San Colombano was the most cytotoxic and the only one to induce the inflammatory response. Particles from Milan and Oasi Bine presented a different behaviour, eliciting effects on viability and ROS formation mainly during summer and autumn. These results can be explained by the chemical properties of particles, which were peculiar for Alpe San Colombano, while Milan and Oasi Bine samples had quite similar composition. In particular, winter PM from Alpe San Colombano resulted to have a higher content of metals with respect to all the other fractions (VESPA project final report). Metals are known inducer of inflammatory effect in cells (Becker et al., 2005; Hetland et al., 2000; Øvrevik et al., 2005; Veranth et al., 2006) and were recently found to increase also cytotoxicity and apoptosis in monocyte cell lines (Jalava et al., 2008). The presence of metals in Alpe San Colombano samples was related to the atmospheric transports since the site was chosen for the absence of local sources of emission. However, transports from the Pianura Padana are unlikely since the sources of emission in this region present low metals levels; moreover the remote site of sampling in winter is located well above the atmospheric mixing layer. The only hypothesis we are considering is that during winter

Alpe San Colombano was influenced by trans-national atmospheric transports from east European regions characterised by high level of metals in their atmosphere.
Other studies investigating the role of sampling site found that the location influences the PM toxicological outcomes, in relation to the specific sources and particles composition (Schins et al., 2002; de Kok et al., 2005; Shi et al., 2006; Steenhof et al., 2011). Moreover the characterization of various urban areas, different in terms of sources of emissions, high traffic, industrial and background areas, revealed that particles collected in the traffic locations were the higher inducers of oxidative and genotoxic potential (Chakra et al., 2007; Boogaard et al., 2011).

The importance of seasonality have been also considered and appeared to be another pivotal characteristic influencing the PM-related effects (Paper I, II and Supplementary data I). Summer PM10 induced cytotoxicity and pro-inflammatory response in the cell lines investigated and this effect has been related to the higher metals and endotoxins content of this fraction (Paper I and II). The higher inflammatory potential of summer particles have been previously reported and related to the higher summer incidence of inflammation-related lung diseases, such as COPD and asthma (Goldberg et al., 2003). Moreover, the role of endotoxins in leading the PM inflammatory effects has been well documented (Kocbach et al., 2008; Hetland et al., 2005) even if the inhibition of this component did not completely block the inflammatory process (Camatini et al., 2010; Gualtieri et al., 2010), thus confirming the contribution of metals.
The winter PMs, on the contrary, induced ROS formation and genotoxic effects, in agreement with data previously reported (Binkova et al., 2003; Chakra et al., 2007). Winter PMs-induced DNA damage was related to the

formation of ROS and to the organic compounds, which were highly represented in these fractions (Paper IV).

Also the particles size influenced the PM toxicological outcomes, since the cytotoxic and pro-inflammatory potential of summer PM10 was typical of this fraction, while it was not present in fine and quasi-ultrafine summer PMs. Various *in vitro* and *in vivo* data showed the higher inflammatory potential of PM10 or coarse fraction (Hetland et al., 2005; Becker et al., 2005; Jalava et al., 2007; Cho et al., 2009). On the contrary, all the winter PMs analyzed had a similar genotoxic effect (Paper I and II), thus surprisingly the size seemed to be of less importance for the effects of winter PM. The high PM2.5/PM10 ratio during the winter season could explain the similar effect of the different fractions; if this is the case, the effects of winter PM10 were related to the fine or ultrafine fractions contained in it. However, a contribution of the coarse fraction, PM10-2.5, cannot be excluded since the effects of this fraction of PM alone were never tested. Moreover the results reported in the Paper IV and Supplementary data section III strongly underlined the importance of particles size, even in the case of a very similar chemical composition: in fact winter PM1 and PM2.5 altered the cell cycle of BEAS-2B cells in a different way, since after 24 h of exposure PM2.5 induced a G2/M increase while PM1 induced a G1 increase. Since the chemical composition of these PMs was similar, this result might be explained by a different bioavailability of the compounds adsorbed which, being related to the surface area, is likely to be higher in smaller particles. This assumption was confirmed by the analysis of the effects promoted by the PM organic fraction (Paper IV and Supplementary data section III): the organic compounds extracted from both PM2.5 and PM1 and diluted in DMSO, therefore easily available for cells in culture, induced a G1 increase after 24h of exposure, similarly to whole PM1.

The last part of the research focused on winter PM2.5, investigating the mechanisms of action that lead to the ROS formation, DNA damage induction and cycle alterations reported in Paper I and II. The gene expression profiling (Paper III) of A549 cells exposed to winter PM2.5 evidenced a modulation of xenobiotics metabolism-related (cytochromes P450, AHRR, TIPARP and ALDH1A3) and pro-carcinogenic (JUN and FOS) genes, that confirmed the genotoxic potential of these particles and suggested a role for the organic components (essentially PAHs). Indeed, PAHs were highly represented in winter PMs, and BaP, used as a reference molecule for pro-carcinogenic PAH, showed genes modulation and DNA damage induction similar to winter PM2.5. Moreover, the activation of PAHs-metabolizing enzymes cytochromes P450 (CYPs) has been also confirmed by protein expression analysis of CYP1B1, one of the member of CYPs enzymes family (Paper III). Other toxicological data reported the genotoxic potential of fine PM, principally originated from combustion processes, (Billet et al., 2008; Bonetta et al. 2009) and suggested a possible relation with the activation of pro-carcinogenic mechanisms, both mutagenic and non genotoxic pathways (Andrysik et al., 2011).

In paper IV the cell cycle alteration was better characterized and the mechanisms underlying it investigated. A G2 cells increase has been reported at a quite early time point (3 h of PM exposure) and this effect was related to the presence of DNA damage, induced by PAHs and ROS as assessed by SCGE assay. However, this G2 arrest/delay was transient since at 10 h it was no more present but an increase of mitotic cells that were prevented to enter in anaphase was reported. The presence of this metaphase arrest indicated the involvement of the spindle assembly checkpoint (SAC), the surveillance mechanism responsible to restrain cells from entering anaphase by preventing the activation of anaphase-promoting complex

(APC), until all chromosomes are properly attached to the bipolar mitotic spindle (Fukasawa, 2008). The involvement of SAC was supported by the morphological analysis of mitotic cells, showing mitotic spindle and centrosomes aberrations (Paper IV), similar to those reported by Glowala et al. (2002) in Chinese hamster fibroblasts exposed to PM10. These data suggested that PM may act as spindle poison, perturbing microtubules (MTs) dynamics. In fact similar spindle morphologies and centrosomes amplification have been reported in cells exposed to notorious spindle poisons as arsenite (Yhi et al., 2006; Liao et al., 2010) and bisphenol-A (Johnson and Parry, 2008); interestingly, both these molecules are present in PM and similar effects on mitotic spindle have been recently reported also for carbon nanotubes (Sargent et al., 2011).

The organic components of winter PM2.5 have been separated from the particles, with the aim to clarify the role of the PAHs. Interestingly, the organic fraction showed an increased potential to induce ROS formation and cell cycle alterations compared to the whole PM, while the washed particles were ineffective. Several studies confirmed the pivotal role of the organics compounds adsorbed on PM (Chackra et al., 2007; Oh et al., 2011; Abbas et al., 2011) and others directly related the PM biological effects to PAHs and nitro- and oxygenated- PAHs (Nel et al., 2001; Binkova et al., 2003; Billet et al., 2008). Here the involvement of PAHs in inducing DNA damage and G2/M accumulation has been investigated treating the cells with CYP enzymes- and AHR-inhibitor α-NF and the reduction of these biological outcomes confirmed the importance of this pathway (Paper IV). DNA damage and G2/M accumulation have been also directly related to ROS formation using the antioxidants NAC and thiourea (Paper IV).

In conclusion the research presented underlines the significant role of PM chemical composition and size in triggering significant biological effects. Summer PM10 has cytotoxic and pro-inflammatory potential while winter PMs are genotoxic and induce alterations of the cell cycle.

The biological elements, bacteria components but possibly also fungi spores and debris, and partly the crustal elements and metals present in summer PM10 are pivotal in determining the biological effects reported. In particular inflammation is strongly related to LPS and metals.

The organic components of particles, in particular PAHs, drive the effects of winter PMs, partly through the induction of DNA damage, mediated by ROS formation, and the activation of DNA damage response. The winter PMs also act as mitotic spindle poisons and this effect, related to the organic compounds adsorbed on particles surface, is even more evident in PM1 when compared to PM2.5. In this case the dimension of particles, in terms of surface available for exchanges, determines the magnitude and/or the timing of the process.

The data reported shed light on significant biological processes triggered by PM and pose the possibility to explain clinical effects evidenced by epidemiological research (TOSCA project final report 2009-2011). At the same time new important questions arise: is it possible to determine which organic compounds are more potent in inducing the biological effects reported for winter PMs, and thus possibly eliminate the sources of these compounds? Is the process here described important for lung cancer development? How is possible to improve the knowledge on the effects of the finest fraction PM0.4, and eventually PM0.1: are new approaches needed, possibly coming from the nanotoxicology experiences? What is the importance of the inflammation triggered by summer PM10 on health outcomes such as asthma, allergic responses and other inflammatory disorder usually increased during the summer season?

Although the research on PM has now decades of experience there are still unresolved questions and others arise thanks to new experimental approaches and techniques. The urban air quality is a matter of concern not only for the population but also for the local administrations that must deal with it and find solutions, and the experimental research will play a central role in giving answers based on data rather than on feelings.

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Paper I

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Season linked responses to fine and quasi-ultrafine Milan PM in cultured cells.

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Running title: size and season linked responses to PM

Abstract

Background: Exposure to urbane airborne particulate matter (PM) is related to the onset and exacerbation of cardiovascular and respiratory diseases. The fine (PM1), and quasi-ultrafine (PM0.4) Milan particles collected during different seasons have been characterised and the biological effects on human epithelial lung A549, monocytes THP-1 cells and their co-culture, evaluated and compared with the results obtained on the PM10 and PM2.5 fractions. *Results:* Chemical composition and transmission electron microscopy (TEM) analysis of PM0.4 showed that this fraction was very similar to PM1 for dimension and biological responses. All the winter fractions increased within 1 h the level of reactive oxygen species (ROS), while only summer PM2.5 had this effect on A549 cells. The phosphorylation of H2AX (γH2AX), a marker of double strand DNA breaks (DSBs), was increased by all the winter fractions on A549 and THP-1 cells while summer PMs did not induced this effect. *Conclusions:* PM0.4 and PM1 biological effects are partly similar and related to the season of sampling, with more potent effect by the winter PMs. The winter PMs damaging effects on DNA correlate with the presence of organic compounds.

Keywords: PM; A549; THP-1; ROS; DNA damage.

1. Introduction

The relationship between the exposure to airborne particulate matter (PM) and the onset of adverse effects on human health has been widely described in many epidemiological studies on the increased rates of mortality and morbidity in the population for cardiovascular and respiratory diseases and lung cancer (de Kok et al., 2006; Englert, 2004; Samet et al., 2000; Sorensen et al., 2003). Pollution-related cardio respiratory effects have been linked to the presence of a pulmonary and systemic inflammatory status, evidenced by the augmented levels of pro-inflammatory cytokines, in the circulating blood of PM exposed population (Pope and Dockery, 2006). *In vitro* studies on various lung cell lines have used different biological endpoints to demonstrate such correlation. It has been demonstrated that PMs from different sources and places can promote the release of inflammatory mediators (Alfaro-Moreno et al., 2002; Hetland et al., 2004), genotoxic effects (Billet et al., 2008; Don Porto Carero et al., 2001; de Kok et al., 2005) and cell death (Alfaro-Moreno et al., 2002; Hsiao et al., 2000).

The structure and composition of PMs are complex and heterogeneous, and influence the biological properties of particles. The chemical composition varies with season and region of sampling, photochemical-meteorological conditions and sources of emissions (Brüggemann et al., 2009; Perrone et al., 2010; Pey et al., 2010). The winter Milan PM chemical composition is characterised by high levels of polycyclic aromatic hydrocarbons (PAHs), whereas summer PM is rich of elements such As, Cr, Cu, Al and Zn (Perrone et al., 2010). These data are rather representative of urban air particles: Brüggemann et al. (2009) reported a higher organic carbon (OC) concentration during winter in comparison with summer PM samples and

explained the presence of such element as the contribution of domestic heating and of a lower atmospheric mixing layer.

PM size is another important parameter influencing biological effects. PM can be classified as PM10 (particles with an aerodynamic diameter less than 10 μm), PM2.5 (Ø<2.5 μm) and PM1 (Ø<1 μm). The fine fractions (PM2.5 and PM1) are dominated by combustion derived particles, consisting mainly of organic and inorganic elements adsorbed onto the surface of a carbonaceous core (Brüggemann et al., 2009; Zerbi et al., 2008). The coarse fraction (PM10) contains a major part of mineral compounds and some adsorbed endotoxins (Pérez et al., 2007; Schins et al., 2004).

The PM coarse fraction (2.5–10 μm) has been associated with pro-inflammatory and cytotoxic effects (Gualtieri et al., 2010; Hetland et al., 2005; Monn and Becker, 1999; Schins et al., 2002). The presence of endotoxins (Soukup and Becker, 2001; Schins et al., 2004; Becker et al., 2005; Camatini et al., 2010) in summer PM10 is partly responsible of the inflammation processes, even the release of inflammatory mediators occurs also in cells exposed to fine PM. This event has been explained with the large surface area of these small particles (Hetland et al., 2004; Donaldson et al., 2001, 2002; Oberdorster, 2001).

The PM fine fraction has been associated mainly to a higher genotoxic potential (Billet et al., 2008; de Kok et al., 2005), with the winter samples being more potent (Binkova et al., 2003; Chakra et al., 2007) in relation to higher PAHs and metal contents. Moreover, positive relationships were found between the formation of reactive oxygen (ROS) and nitrogen (RNS) species and the induction of DNA damage (de Kok et al., 2006; Gualtieri et al., 2011). Depending on the amount formed, ROS may result in necrosis as

well as apoptosis. The most severe DNA lesions produced by ROS are double strand breaks (DSB; Mills et al., 2003), which may be detected by the phosphorylation of histone 2AX (γH2AX; Albino et al., 2009).

Besides the increasing epidemiological data on particles with a diameter less than 1μm (quasi-ultrafine), there are still few data on the chemical composition and biological effects of the ultrafine fraction (Ø<0.1 μm) (Kudo et al., 2011; Chen et al., 2010) for the difficulty in sampling such particles (Furuuchi et al., 2010).
In order to fulfil this gap of knowledge on the biological responses triggered by the finest PM fractions on human cell lines, we extended our study to the effects produced by Milan summer and winter quasi-ultrafine PM0.4 and PM1 on A549 and THP-1 cells and on the co-culture of these two lines. Cell viability, pro-inflammatory proteins release, ROS production and DNA DSB induction are evaluated and compared with the effects produced by the other PM fractions.

2. Materials and Methods

2.1. PM sampling, physical and chemical characteristics

PM particles were collected at Milan Torre Sarca, a representative background site for air quality, during summer (June and July) and winter (January and February) of 2008-2009. A low volume gravimetric sampler (EU system, FAI Instruments, Rome, Italy) was used to collect PM samples on Teflon or quartz filter for biological analysis and chemical characterisation respectively. Particles were characterised for elements (mineral dust and trace elements), polycyclic aromatic hydrocarbons (PAHs), inorganic ions and total carbon as previously described (Perrone et al., 2010; Camatini et al., 2010; Gualtieri et al., 2009).

2.2. Particle morphological characterisation

Particles to be used for biological experiments were extracted from Teflon filters by sequential sonications (SONICA, Soltec, four cycles of 20 min each) in sterile water. Particle suspensions were dried into a desiccator, weighed and stored at -20°C until use and re-suspended in sterile water at a final concentration of 2 μg/μl,just before use. Extracted particles (PM0.4 and PM1) were prepared for TEM as reported (Gualtieri et al., 2009). Briefly, aliquots of resuspended particles were pipetted onto Formvar® coated 200mesh copper grids and air-dried. Grids were analysed by a Jeol JEM-1220 TEM equipped with a CCD camera and digital images taken at a magnification of 20k. A total of 300 particles for each sample were measured and particle size distribution was obtained.

2.3. Endotoxin content and microbiological molecular characterisation

Endotoxin content in PMs was determined by a quantitative chromogenic Limulus Amebocyte Lysate (LAL) test (International P.B.I. S.p.A., Italy) following the manufacturer's instructions. Briefly, PM extracts were diluted in apyrogenic water to the concentration of 10 μg/ml, mixed with pyrochrome LAL reagent and incubated at 37°C for 40 min. The reaction was then stopped with 5% acetic acid and the absorbance of the samples was determined by a spectrophotometer (Ascent scan multiplate reader, Thermo Scientific) at 405 nm. The concentration of endotoxin was calculated from a standard curve of LPS (*E. coli)*. The endotoxin concentrations were expressed as endotoxin units per milligram (EU/mg) of tested particles.

The molecular characterisation of the microbial community associated to a representative summer PM10 sample collected in September 2009 was carried out by 16S rRNA-tag sequencing using Illumina GA IIx. Details of

the experimental procedures and sequence data are provided as Supplementary Data.

2.4. Cell culture and treatment.

The human lung type II pneumocytes, A549 cell line, and the human monocytes, THP-1 cell line (ATCC, Rockville, MD, USA), were maintained in Opti-MEM medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS), 100 μg/ml penicillin and 100 U/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37°C. A549 were seeded at a concentration of 70.000 cell/well in 12 well-plates (or 150.000 cells/well in 6 well-plates). The next day, 80% sub confluent cells were treated with 10 μg/cm^2 of PM. THP-1 cells (150.000 cells/well) were seeded in 12 well-plates and immediately treated with 10 μg/cm^2 of PM. Co-culture experiments were performed in 12 well-plates, THP-1 (150.000 cells/well) were added 24 hours after seeding A549 cells and treatment performed afterwards. For each experiment, untreated control cells were carried together.

2.5. Cytotoxicity

Cell membrane integrity was used as a marker of cytotoxicity and measured after 24 h of treatment by LDH release in the culture medium. The LDH colorimetric assay (TOX-7, Sigma Aldrich; Italy) was performed following the manufacturer's instructions: 100 μl of culture medium were added to 200 μl of LDH substrate and incubated at room temperature for 30 min in the dark. The reaction was terminated by the addition of 30 μl of 1N HCl to each well. Samples were read by the Multiscan Ascent multiplate reader at 490 nm.

2.6. Cytokine release

After 24 h of treatment, cell culture media were collected and centrifuged for 10 min at 250 g to remove cell debris and floating particles. The final supernatants were stored at -70°C until use. IL-6, IL-8 and IL-1β protein levels were determined by ELISA (Cytosets, BioSource International) according to manufacturer's guidelines. The cytokines were quantified by a Multiskan Ascent multiplate reader (Thermo Scientific, Inc.).

2.7. ROS production

A549 and THP-1 cells were incubated at 37°C with 5 μM of 2'7'-dichlorodihydrofluorescein diacetate (DCFH) in PBS for 20 min. Then cells were washed in PBS, treated with PM for 1 h, harvested and suspended in 500 μl of PBS. The ROS production, detectable by the oxidation of DCFH to dichlorofluorescein (DCF), was quantified by measuring the fluorescence intensity of 10,000 events with the cytometer EPICS XL-MCL (Beckman-Coulter) using 525 nm band pass filter. Data were analyzed using the EXPO32 ADC software (Beckman-Coulter).

2.8. DNA double strand breaks

γH2AX was used as marker of double strand breaks and measurements were performed by flow cytometry. Cells were harvested after 24 h of treatment, fixed with 1% paraformaldehyde on ice for 15 min, re-suspended in cold methanol 90% and stored overnight at -80°C prior to analysis. After discharge of ethanol, cells were washed once in PBS 0.5% BSA and incubated 4 h with an Alexafluor-488 conjugated γH2AX antibody (1:100 dilution, Cell Signaling) in PBS 0.5% BSA, 0.2% Triton X-100 at room temperature. Finally, cells were washed once in PBS 0.5% BSA, resuspended in 500 μl PBS and analyzed on the Beckman Coulter EPICS

XL-MCL flow cytometer. Fluorescence of 10.000 events was detected using 525 nm band pass filter.

γH2AX expression in A549 cells was analysed with a immunocytochemical technique too. Briefly, cells grown on cover slips were treated with PM as described above and with etoposide (1.65 μM), as positive control, for 24 h. After washed in PBS, the samples were fixed with 1% paraformaldehyde for 15 min on ice and incubated in PBS 0.5% BSA, 0.2% Triton X-100 for 15 min at room temperature. γH2AX was then labelled with the anti- γH2AX Alexafluor-488 conjugated antibody (Cell Signaling) diluted 1:100 in PBS 0.5% BSA, 0.2% Triton X-100, for 4 h at room temperature. DNA was counterstained with DAPI and slides observed by a Leica fluorescence microscope and digital images were taken.

2.9. Statistical analyses

Statistical differences between samples were tested with one-way ANOVA and post hoc comparisons performed with Dunnett's method, by using SigmaStat 3.1 software. Statistical differences were considered to be significant at the 95% level ($p < 0.05$).

3. Results

3.1. Morphological characterisation of extracted particles

PM composition resulted strongly related to the season of sampling. Water-soluble inorganic ions were the chemicals most abundant in all PM fraction; nitrate (NO_3^-) was the most abundant ion in winter season while sulphate (SO_4^{--}) was prevalent in summer (data not shown). Moreover summer fractions were enriched in metals while winter PMs were enriched in PAHs.

PM1 and PM0.4 had identical composition, within the same season and the content of elemental carbon was 14.6 by mass percentage in summer and

11.8% in winter and organic carbon 30% in summer and 35% in winter. The content of elements was 2.8% in PM1 and 0.8% in PM0.4 summer samples and 0.5% in PM1 and 0.7% in PM0.4 winter samples. PAHs content was ten times higher in winter in comparison with summer sample. The PAHs concentration in winter PM0.4 and PM1 was 450 ng/mg and PM1 of 650 ng/mg respectively (Bolzacchini et al., personal communication).

Figure 1 illustrates the PM0.4 (a) and PM1 (b) morphology as seen at TEM. Particles extracted from Teflon filters appeared as aggregates of smaller round-shaped nanoparticles of 20–40 nm. The histograms of particle size distribution (Fig. 1c-d) highlighted a slight dimensional difference between these two fractions, constituted mainly by aggregates with a dimension variable from 20 nm to 160 nm.

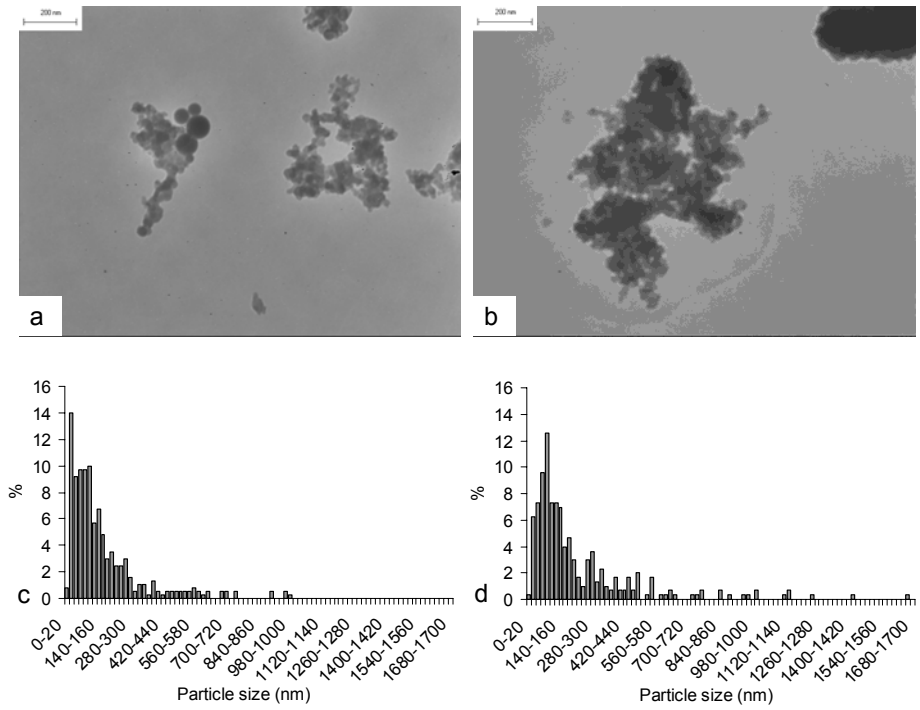


Fig. 1: Ultrastructural morphology of PM0.4 (a) and PM1 (b). The histograms describe the particle size distribution of PM0.4 (c) and PM1 (d).

3.2. Endotoxin content and microbiological molecular characterisation

Endotoxin levels in PM samples, measured with the LAL test, are presented in Figure 2a. The endotoxin content is higher in the summer PMs in comparison with the winter ones. Apparently the endotoxin quantity was related to the particle dimension since the highest level was measured in summer PM10. Concordantly the molecular characterisation of the bacterial fraction evidenced that the gram-negative bacteria phylum was the dominant airborne community in summer PM10 samples (Fig. 2b).

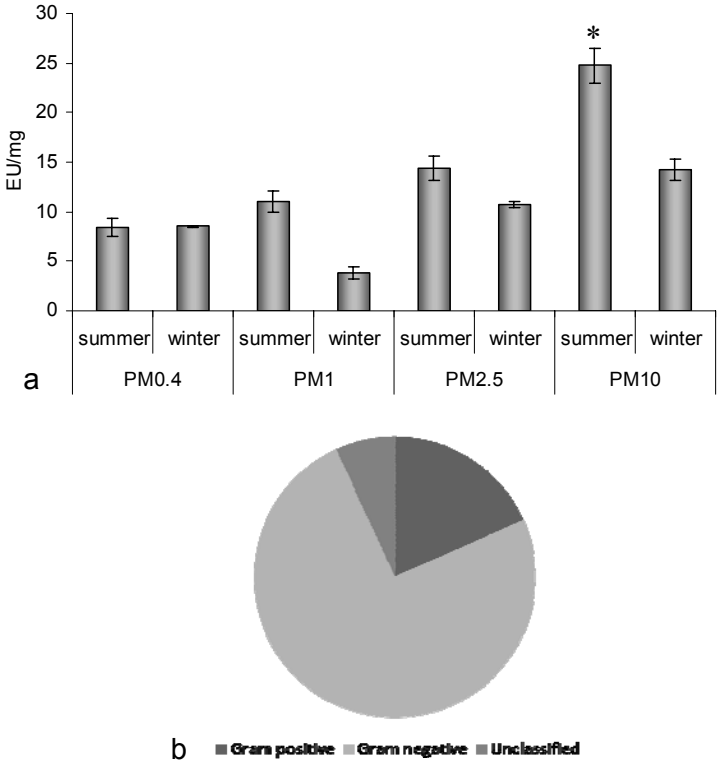


Fig. 2: a) Endotoxin content of winter and summer PMs, as determined by LAL test. Data are mean ± SEM of independent experiments (n=3). * Statistically significant difference from the other samples, $P<0.05$. b) Relative abundances of Gram-negative and Gram-positive bacteria, classified at phylum level by Ribosomal Database Project Classifier.

3.3. Cytotoxicity

PM0.4 and PM1 did not induce significant cytotoxic effects (Fig 3), while summer PM10 significantly increased LDH release both in THP-1 cells (Fig 3a) and co-culture system (fig.3b). Winter PM2.5 and PM10 were cytotoxic only to THP-1 cells (Fig.3a). Cytotoxicity was never observed in A549 cells treated with the PM fractions tested (data not shown).

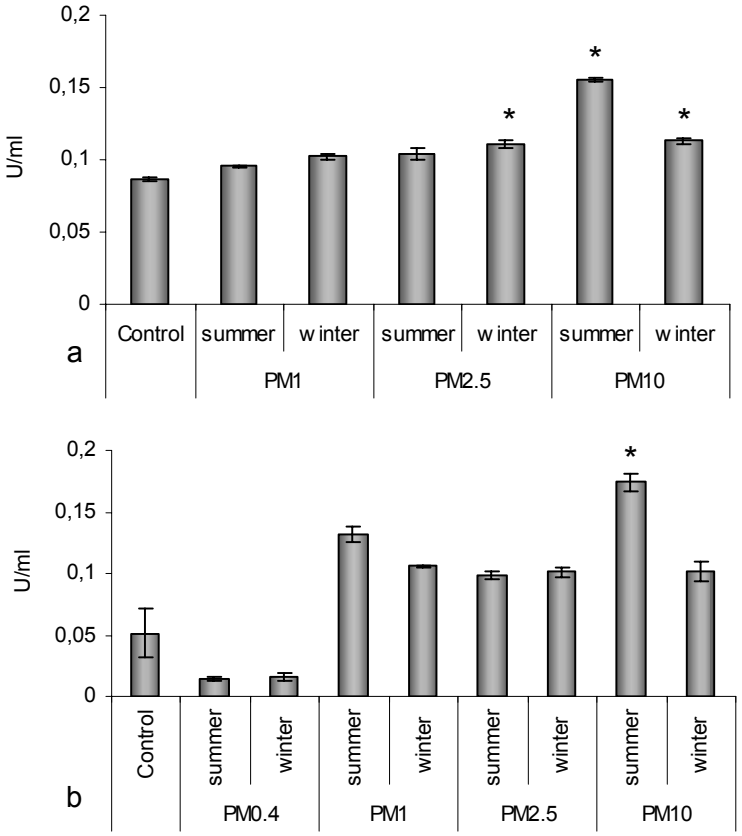
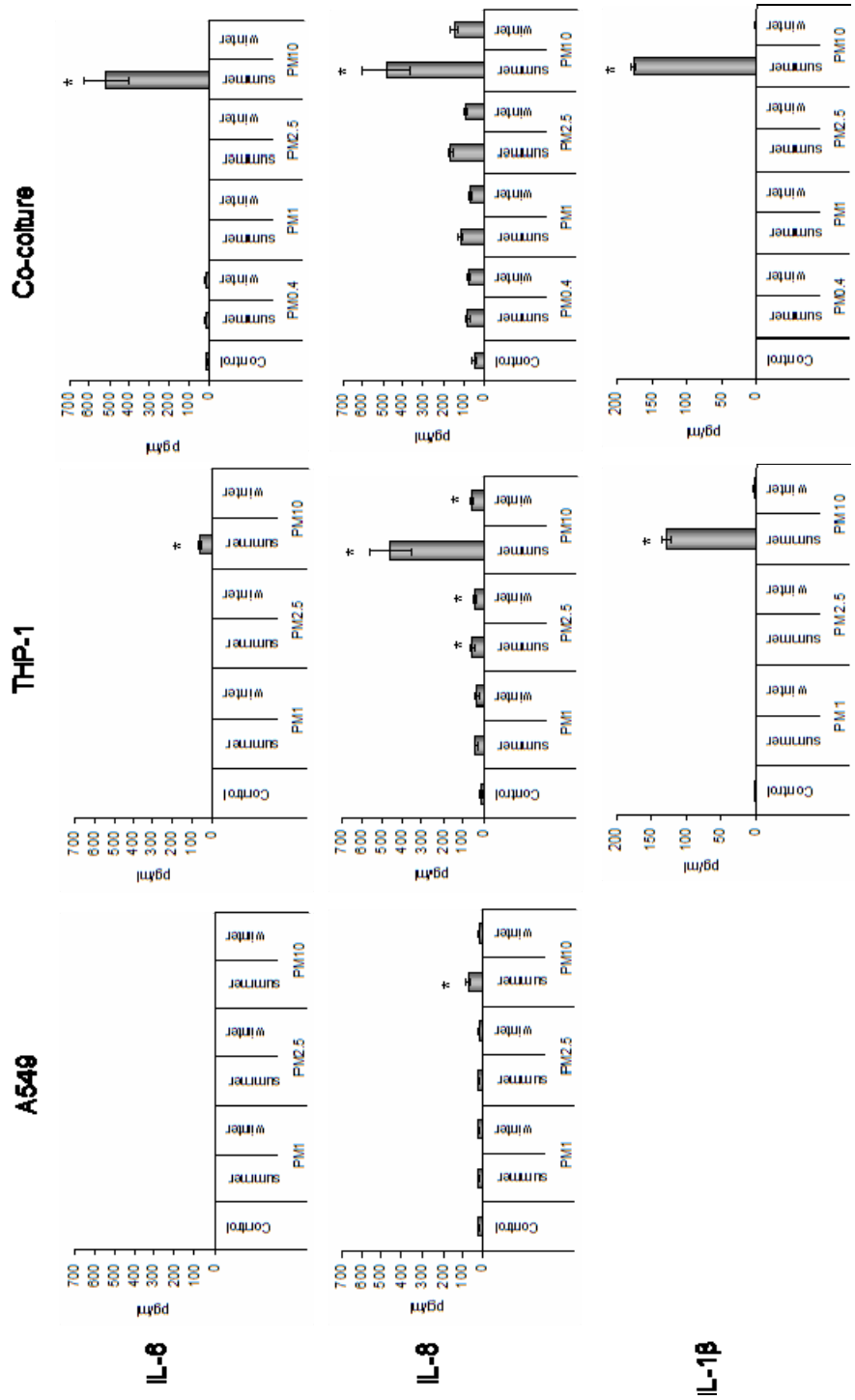


Fig. 3: LDH release in THP-1 cells (a) and in co-culture system of A549 and THP-1 cell lines (b) exposed for 24 h to 10 μg/cm^2 of PM. The data are mean ± SEM of independent experiments (n=3). * Statistically significant difference from unexposed cells (control), P< 0.05.

3.4. Cytokine release

The release of cytokines, measured in the culture medium to analyze the presence of an inflammatory response, was performed analysing IL-6, IL-8 and the systemic cytokine IL-1β. The results (Fig. 4) showed that PM0.4 and PM1 did not induce significant inflammatory effect in none of the cell system analysed. IL-1β release was significant in THP-1 and co-culture system treated with summer PM10. The summer coarse fraction triggered also the release of IL-6 and IL-8, as previously described (Gualtieri et al., 2010; Camatini et al., 2010). The release of IL-6 was much higher in the co-culture system (514.8 pg/ml) in comparison with THP-1 cells (60.1 pg/ml) (Fig.4).

Fig. 4: Cytokines release in A549 and THP-1 cell lines in mono- and co-culture system exposed for 24 h to 10 μg/cm^2 of PM. The data are mean ± SEM of independent experiments (n=3). * Statistically significant difference from unexposed cells (control), $P < 0.05$.


A549
THP-1
Co-coture
IL-8
IL-8
IL-1β
pg/ml
Control
summer
winter
PM0.4
PM1
PM2.5
PM10
*

3.5. ROS production

Flow cytometry analyses were used to quantify the level of ROS in A549 and THP-1 cells exposed for 1 h to 10 μg/cm^2 of PMs. Figure 5 shows the intensity of fluorescence of the dye DCF, responsive to ROS presence, in A549 cells. All winter PMs induced a significant and comparable increase of ROS: winter PM0.4 and PM1 exposed cells reported an fluorescence intensity of 5 and 5.4 respectively versus the 3.8 fluorescence a.u. of control cells (Fig. 5). Summer PMs, with the exception of PM2.5, did not induce ROS formation. Similar results were obtained for THP-1 cells (data not shown).

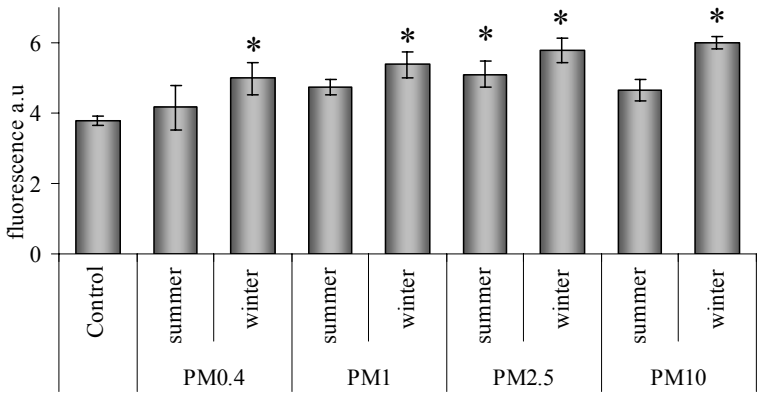


Fig. 5: ROS production in A549 cells exposed for 1 h to 10 μg/cm^2 of PM. The data are mean ± SEM of independent experiments (n=4). * Statistically significant difference from unexposed cells (control), P< 0.05.

3.6. DNA double strand breaks

The presence of DSBs was evaluated by the quantification of the phosphorylation of the histone 2AX (γH2AX; Fig. 6).

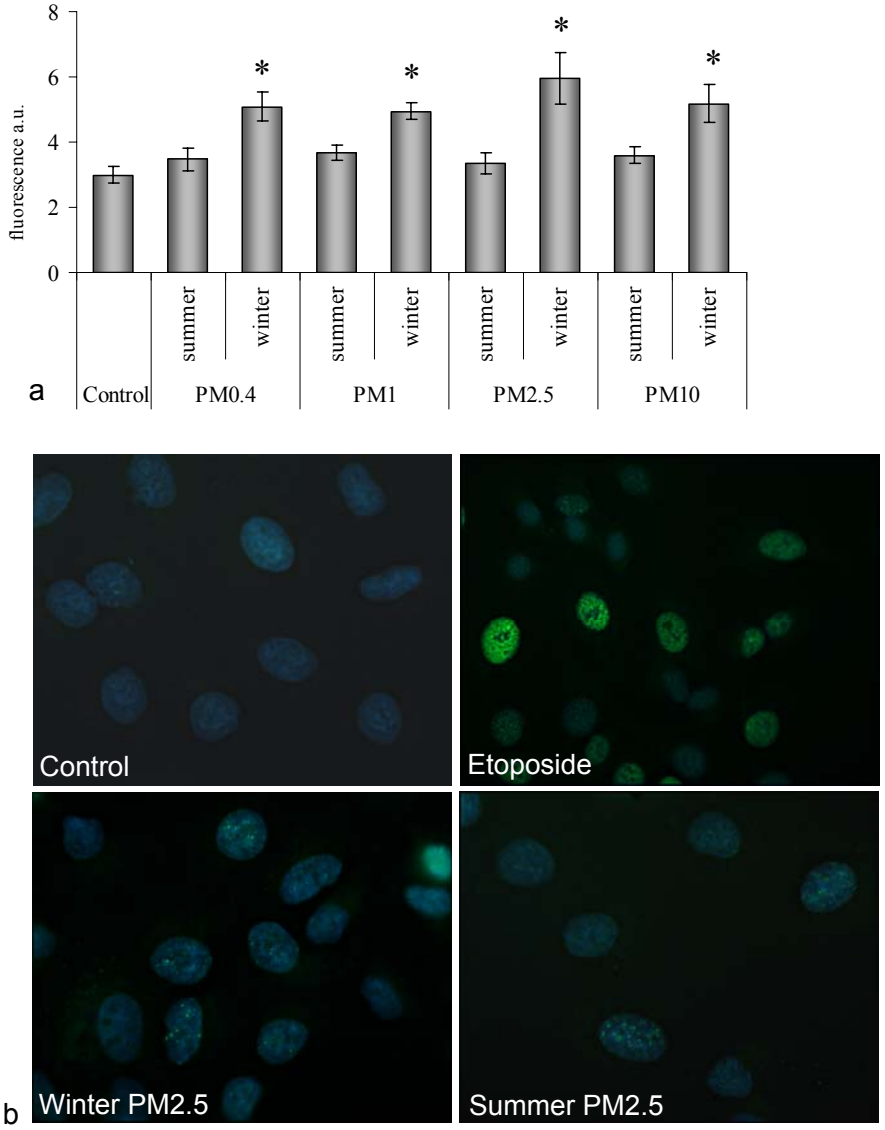


Fig. 6: γH2AX expression in A549 cells exposed for 24 h to 10 μg/cm^2 of PMs. a) The histogram shows the fluorescence values (arbitrary units; a.u.) measured with flow cytometry. Data are presented as mean ± SEM of independent experiments (n=4). * Statistically significant difference from unexposed cells (control), $P< 0.05$. b) Fluorescence microscopy shows γH2AX distribution in control cells and cells treated with etoposide (1.65 μM), summer and winter PM2.5.

A significant increase in γH2AX expression was induced by all winter PMs with a similar intensity (Fig. 6a): PM0.4 exposed A549 cells registered a fluorescence intensity of 5.1 a.u. and versus 2.98 a.u. in controls.No effect was induced by summer PMs and similar results were obtained for THP-1 cells (data not shown).

Fluorescence microscopy (Fig. 6b) evidenced the presence of the characteristic distinct foci of γH2AX in the chromatin of cells treated with winter PM2.5 and, to a lesser degree, with summer PM2.5. γH2AX fluorescence in untreated control cells was negligible.

4. Discussion

Recently great attention has been devoted to the urban fine and ultrafine PMs, since epidemiological and clinical studies have reported that this PM fractions have a higher potential to cause vascular and cardiac dysfunction in humans and animals (Cho et al., 2009; Cozzi et al., 2006; Gong et al., 2003; Hwang et al., 2008; Mills et al., 2009). It has been recently reported that in Milan the personal exposure to ultrafine particles is as high as in other very polluted cities like Athens (Cattaneo et al., 2009). The data reported for Milan are however related to the spring season, when the mean concentrations of PM are usually lower than in winter. It is thus reasonable to expect even higher concentration of UFPs in Milan during the winter season when the safety threshold of 50μg/m^3 for PM10 is largely exceeded. Summer and winter Milan PM1 and PM0.4 have been for the first time sampled and analysed to evaluate their physico-chemical and biological properties. These fractions resulted to be very similar in the same season. The main difference between summer and winter fine and quasi-ultrafine

PMs was the content in PAHs which was higher in winter, for differences in the photochemistry of the atmosphere, as suggested by Kudo et al. (2011).
The morphological characterization showed that fine and quasi-ultrafine PMs were mainly constituted by round-shaped nanoparticles of 20-40 nm in diameter, which formed aggregates ranging from 20 nm to 160 nm. Milan winter PM2.5, previously analyzed (Gualtieri et al., 2009), was represented by particles of 40 nm which aggregated in 300 nm structures. Thus, although the agglomerates of fine and quasi-ultrafine particles are different in dimension, their chemical characterization confirmed that the main sources were carbon soot particles generated by combustion processes.
The biological responses of PM1 and PM0.4 from the same season were similar. None of these fractions were able to trigger an inflammatory responses, which instead was produced by the summer coarse fraction with the release of significant quantity of IL-6, IL-8 and IL-1β. In agreement with our previous observations (Gualtieri et al., 2010), A549 cells presented only a slight increase in IL-8 release, while the here investigated THP-1 cells and co-culture system presented a significant release of all the cytokines; interestingly, the co-culture system was more sensitive to the PM treatment in comparison with the mono-cultures, and the IL-6 release was dramatically augmented. These results are in agreement with those of Wottrich et al. (2004) who showed a high increase in IL-6 and IL-8 levels in A549/THP-1 and A549/Monomac6 co-cultures exposed to hematite and silicasol particles. This higher release in co-colture system may depend on the contact between THP-1 and A549 cells, which can induce THP-1 to express macrophages-like properties (Wottrich et al., 2004; Striz et al., 2001). Noteworthy of mention is the ability of the summer PM10 to induce a significant release of IL-1β, a potent promoter of inflammatory stimuli (McColl et al., 2007; Fujii et al., 2001).

The data of Cho et al. (2009) showing the *in vivo* effects of different PMs producing a higher pulmonary inflammation of the coarse fraction and cardiac injury potency for the ultrafine one, are in line with our results. The effects on the cardiovascular system are usually related to the translocation of UFPs insoluble fraction from the lung (Furuyama et al., 2009), dissolution of soluble metals, which in turn affect endothelial cells function (Li et al., 2010), and noteworthy, to the ability of UFPs to increase the levels of ROS (Brook et al., 2010). In fact all the winter PM fractions here examined resulted to be potent ROS inducers and promoters of the H2AX phosphorylation. The ROS increase could be related to the soluble metals as well as to the presence of PAHs and quinones. In fact, winter Milan PM contains a quantity of PAHs 10 fold higher than that of summer samples (Perrone et al., 2010; Gualtieri et al., 2010; Camatini et al., 2010) and a direct correlation between ROS generated by PM and PAHs content has been reported (de Kok et al., 2005). ROS production is promoted by PAHs usually through the cytochrome P-450 activation, which transforms PAHs in reactive molecules, diones, as previously demonstrated in BEAS-2B cells (Reed et al., 2003). The most severe ROS damage is its interaction with DNA, and this event has been here assessed by evaluating the phosphorylation of the histone 2AX (γH2AX), marker of DNA DSBs (Mills et al., 2003; Lips and Kaina, 2001).

Winter PM1 and PM0.4 caused a significant increase in γH2AX expression as well as PM2.5 and partly PM10. Delfino et al. (2010) stated that the PAHs in fine and quasi-ultrafine PMs are the major redox-active component. As previously reported for diesel exhaust particles (Riedl et al., 2005) PAHs and quinones trigger the formation of ROS which may promote oxidative injury and inflammatory response through the activation of the nuclear factor-kB (NF-kB). Our data clearly outline the higher potency of PAHs rich PMs to promote ROS formation. The lack of inflammatory response may be due to

the high oxidative damage triggered by the winter PMs which determine DNA damages and subsequent cell cycle arrest as previously reported in Longhin et al. (2010). The potency of PM0.4 and PM1 to increase the intracellular ROS level needs attention. The dimensions of such fractions consents the passage through the alveolar capillary barrier and the interaction with endothelial cells. Oxidative stress and pro-inflammatory responses in endothelial cells exposed to ultrafine PMs have been reported (Li et al., 2010; Brook et al., 2010) and increased ROS at endothelial level might promote the formation of NO, which is a potent modulator of the vascular functions (Arenas et al., 2005).

In conclusion, our results demonstrate that PM0.4 biological effects are similar to the ones produced by PM1, and this result may depend by the rather similar particles size during experimental conditions. Interestingly, the A549/THP-1 co-culture system showed a significant high level of IL-6, less evident in the THP-1 monoculture. All the winter fractions, with a high quantity of PAHs, had a key role in eliciting ROS formation and DNA damage. Finally, our data confirm that PM inhalation needs to be further investigated for the understanding of the role of ultrafine particles in eliciting cardiovascular diseases.

Conflict of interest statement

Nothing to declare

Aknowledgements

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Paper II

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Biological effects of Milan PM: the role of particles dimension and season of sampling

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Abstract

The biological effects induced in human pulmonary cells by atmospheric particles collected in Milan have been analysed according to their dimension and season of sampling. Summer and winter PM_1, $PM_{2.5}$ and PM_{10} were chemically characterized and used for exposure of pulmonary cultured cell lines. Cell viability, proinflammatory cytokine expression, cell cycle modifications and cytochrome P450 CYP1B1 expression were analysed. Chemical characterization showed that summer fractions had a higher endotoxin content and were enriched in metals while winter PMs in PAHs. In line with this aspect, summer PM_{10} induced stronger cytotoxic and inflammatory effects in comparison with the other fractions. Winter fine PMs provoked a cell cycle arrest in G_2/M phases and remarkable cytochrome P450 CYP1B1 activation, suggesting a potential genotoxic effect.

1. Introduction

Particulate matter (PM) has been unanimously recognized as a hazardous air pollutant whose effects on human health are well known. All the existing epidemiological data agree in describing consistent associations between high PM concentration and increase in mortality and morbidity in the population (Englert, 2004). The Po Valley, and particularly the city of Milan, present high concentrations of air pollutants due to their geomorphological and meteorological characteristic. In Milan the annual mean of PM_{10} is measured in 56 $\mu g/m^3$, with maximum daily peaks over 200 $\mu g/m^3$ (ARPA Lombardia, 2006), clearly exceeding the annual and daily mean EU limits of 40 $\mu g/m^3$ and 50 $\mu g/m^3$ respectively (EU air quality directive 99/30/CE). PM is a complex mixture of particles with different size and chemical composition. According to the criteria of particle size, it can be classified as PM_{10} (particles with an aerodynamic diameter less than 10μm), $PM_{2.5}$ (Ø<2.5μm) and PM_1 (Ø<1μm). The fine fractions ($PM_{2.5}$ and PM_1) are dominated by combustion derived particles, consisting of organic and inorganic compounds adsorbed onto the surface of a carbonaceous core (Zerbi et al., 2008). The coarse fraction (PM_{10}) contains a major part of mineral compounds and some adsorbed elements, such as endotoxins (Pérez et al., 2007). Seasonality is another critical parameter for particle chemical composition, due to the different photochemical and meteorological conditions and to the kind of human emissions. The different toxic responses are supposed to be linked to the PM chemical characteristics. For these reasons, the aim of this research is to evaluate the toxicity of Milan PMs, by a comparative study of summer and winter PM_1, $PM_{2.5}$ and PM_{10}, to highlight possible differences in the mechanisms of action that ultimately will be helpful in understanding the effects on human health. After being chemically characterized, the PM fractions were tested on human pulmonary

cell lines to analyse PM-related effects on cells. Since the mechanisms elicited by the interaction between particles and pulmonary cells remain an uncertain question, a first attempt to study toxicity pathways was done, by evaluating the expression of the cytochrome P450 (CYP1B1).

2. Methods

2.1. PM sampling and preparation

PM_1, $PM_{2.5}$ and PM_{10} samples were collected at the urban site Torre Sarca (Milan), and processed for cell culture experiments as previously reported (Gualtieri et al., 2009).

2.2. Chemical characterization

PM samples were chemically characterized for inorganic ions, elemental and organic carbon, elements (mineral dust and trace elements), and polycyclic aromatic hydrocarbons (PAHs) as reported in Bolzacchini et al., 2002. Endotoxin content was determined by Limulus amebocyte lysate (LAL) test accordingly to manufacturer instructions (Associates of Cape Cod, Inc., East Falmouth, MA).

2.3. Cell culture and treatment

The human lung epithelial carcinoma cell line, A549, and the human acute monocytic leukemia cell line, THP-1 (American Tissue Type Culture Collection, ATCC, Rockville, MD, USA) were routinely maintained in OptiMEM medium at pH 7.2, 5% CO_2 and 37°C. A549 cells were treated after 24 hours from seeding with 10 μg/cm^2 of summer and winter PMs. THP-1 cells were treated immediately after seeded with 10 or 25 μg/cm^2 of summer and winter PMs.

2.4. Cell viability, inflammatory response, cell cycle and cytochrome expression

Particles exposure was extended for 24 hours before performing experiments. Cell viability was determined by lactate dehydrogenase (LDH) assay that provides an indirect evaluation of cell membrane integrity. LDH release in the culture medium has been evaluated accordingly to manufacturer instructions (Lactic Dehydrogenase Assay Kit, Sigma-Aldrich, Inc., St Louis, MO, USA). Cytokine release is a marker of pro-inflammatory response in cells. Interleukine IL-6, IL-8 and IL-1β levels were determined in culture medium by sandwich ELISA according to the manufacturer's guidelines. Absorbance was measured by a Multiskan Ascent multiplate reader (Thermo Scientific, Inc.). Cell cycle analysis were carried out to investigate genotoxicity in cells exposed to PMs. The fluorescent dye propidium iodide (PI) was used to stain DNA of cells and fluorescence was measured by EPICS XL-MCL (Beckman-Coulter) flow cytometer. Quantitative western blot analysis were performed to determine cytochrome P450 CYP1B1 expression (primary CYP1B1 polyclonal antibody, Santa Cruz). Benzo[a]pyrene 14μM was used as positive control. Densitometric quantification was performed with densitometer GEL DOC 2000 (BIO-RAD).

3. Results

3.1. PM characterization

PM composition is strongly affected by seasonality. Water-soluble inorganic ions were the major chemical fraction of PM, Nitrate (NO_3^-) was the most abundant ion in winter season, while sulphate (SO_4^{--}) was prevalent in summer (data not shown). Moreover summer fractions were enriched in metals while winter PMs showed a greater contribution of PAHs (Table 1).

Summer PMs presented endotoxin content higher than the winter ones (Table. 1). Furthermore endotoxin content was directly related to the PM fraction analysed, since PM_{10} showed the highest values, while PM_1 the lowest.

	PM_1		$PM_{2.5}$		PM_{10}	
	Summer	Winter	Summer	Winter	Summer	Winter
PAHs	0.02±0.00	0.37±0.01	0.04±0.00	0.16±0.01	0.03±0.00	0.21±0.02
Elements	2.85±1.52	0.53±0.24	2.26±0.65	0.89±0.52	4.77±1.8	2.52±0.75
Endotoxins	13.6±0.63	2.8±0.15	17.6±0.22	11.29±0.03	25.9±0.63	16.87±0.91

Table 1 Chemical characterization of PM fractions. Polycyclic aromatic hydrocarbons (PAHs) and elements (Al, As, Ba, Cd, Cr, Cu, Fe, Mn, Mo, Ni, Pb, V, Zn) values are expressed as mean mass percentage ± SD. Endotoxin content is expressed as mean EU/mg ± SD.

3.2. Cell viability

Viability, analysed by LDH test, was not affected in A549 cells treated with summer and winter PMs at 10μg/cm^2 (Fig.1A), while it was significantly reduced in THP-1 cells exposed to PM_{10} (especially summer) and winter $PM_{2.5}$ (Fig. 1B).

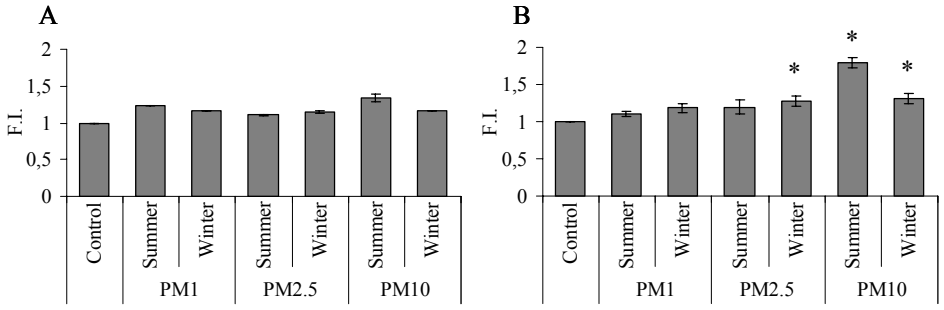

Fig. 1: A549 (A) and THP-1 (B) cell viability measured by LDH release after 24h exposure to 10μg/cm^2 of PM fractions. Data are expressed as fold increase with respect to control. * Statistical different from control at $p<0,05$.

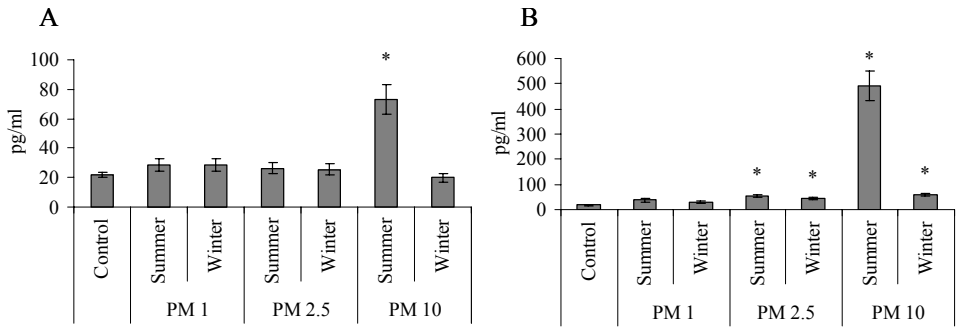

Fig. 2: IL-8 release in A549 (A) and THP-1 (B) cells after 24h exposure to 10µg/cm^2 of PM fractions. * Statistical different from control at p<0,05. Note the differences in the Y scales.

3.3. Inflammatory response

In A549 cells, IL-6 and IL-1β production did not increase after treatment with any PM fraction (data not shown), while IL-8 was slightly but significantly increased by summer PM_{10} exposure (Fig. 2A). In THP-1 cells, IL-8 registered a significant increase after exposure to summer and winter $PM_{2.5}$ and PM_{10} (Fig. 2B). In particular, summer PM_{10} triggered the major response inducing IL-8 increase up to 492pg/ml versus 17.4pg/ml of controls. IL-6 and IL-1β behaved similarly, with significant induction after summer PM_{10} treatment, which showed values of 58.7 and 127.5pg/ml respectively versus 0.3 and 0.9pg/ml in controls (data not shown).

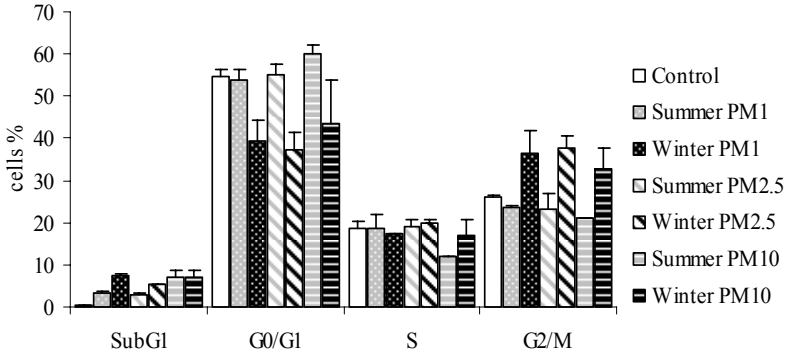

Fig. 3: THP-1 cell cycle after 24h exposure to 25µg/cm^2 of PM fractions.

3.4. Cell cycle

A549 cells exposed to 10µg/cm^2 PMs did not show altered cell cycle, while THP-1 did, with a slight decrease in S-phase cell percentage after summer PM_{10} treatment and an increase of cell percentage in G_2/M phases after exposure to winter PM_1 (data not shown). In order to strengthen these findings, THP-1 cells were exposed to a higher PM dose (25µg/cm^2). As evidenced in Fig. 3, all winter PMs caused a cell cycle arrest in G_2/M phases. Concomitantly, the effect of summer PM_{10} was amplified at this dose of treatment, with cells in S-phase falling to 12% from 18.7% of control.

	Control	Summer $PM_{2.5}$	Winter $PM_{2.5}$	BaP
CYP1B1	0.52	2.81	3.18	4.81
Tubulin	0.58	0.61	0.58	0.61

Table 2 Quantitative western blot analysis of cytochrome P450 CYP1B1 expression in A549 cells exposed to 10µg/cm^2 of summer and winter $PM_{2.5}$ and to Benzo[a]pyrene (BaP) 14µM. Values are reported as ODu/mm^2.

3.5. Cytochrome expression

Since PAH-enriched winter PMs seemed to induce genotoxic effects, the expression of the enzyme CYP1B1, involved in the metabolism of such

compounds, was analyzed. As shown in Table 2, densitometry of western blot gave positive results, revealing the presence of a major quantity of CYP1B1 in cells exposed to winter $PM_{2.5}$ when compared to both control and summer $PM_{2.5}$.

4. Discussion

Summer and winter PM fractions showed a deep difference in chemical composition and endotoxin content, the latter being higher in summer PM_{10}. These findings can explain the different toxic effects observed. Indeed summer PM_{10} revealed a higher capacity of triggering cytotoxic and pro-inflammatory effects both on THP-1 and A549 cells, since it induced cell death and interleukin release. Other experimental and epidemiological studies suggest that the coarse fraction may have higher pro-inflammatory potential than the fine ones (Camatini et al., 2010; Hetland et al., 2005). Moreover it has been demonstrated that summer and springtime PM induced stronger cytokine responses than winter PM in primary alveolar macrophages and RAW 264.7 cells (Hetland et al., 2005). Endotoxins have been associated with the inflammatory effects of PM both *in vitro* and *in vivo* and it has been demonstrated that the IL-8 release induced by summer PM_{10} was significantly mediated by endotoxins (Gualtieri et al., 2010; Camatini et al., 2010). Moreover chemical characterization showed that PM_{10} contained more crustal elements than fine fractions, and these compounds also can contribute to the inflammatory potential of this PM fraction (Øvrevik et al., 2005). On the other side winter PMs showed a potential genotoxic effect on both THP-1 and A549 cells with cell cycle arrest in G_2/M phases and increased expression of cytochrome CYP1B1 as major events. Gualtieri et al. (2010) already reported an increase in mitotic-arrested BEAS-2B cells after fine PM exposure, supporting the present

results obtained in THP-1 cells. Cell cycle arrest has been associated with DNA damage (Huang et al., 2005), that is likely associable to the relatively high PAH concentration in winter fine PMs. Indeed, PAHs are notorious inducers of DNA-adduct and oxidative DNA damages. Since A549 cells have been reported to express inducible forms of cytochrome P450 family proteins, the increased expression of cytochrome CYP1B1, caused by winter PMs, can be considered a valuable molecular marker of exposure. This study showed that the variability of biological effects induced by PM from Milan depends primarily on the season of sampling while the aerodynamic diameter of particles has a lower impact. Summer PM_{10} was more cytotoxic than the other sampled fractions, although winter fine PMs triggered cell cycle arrest in G_2/M phases and cytochromes P450 activation. The perspectives of this research are oriented to the analysis of the possible involvement of toll-like receptors (TLR-2/TLR-4) in summer PM_{10}-induced inflammation. A further investigation will involve the role of the cell cycle regulatory proteins (Chk and CDK) and the mitotic spindle machinery to explain the winter PMs-induced cell cycle alteration.

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Paper III

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Gene expression profiling of A549 cells exposed to Milan PM2.5

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Running title: Genotoxicity of fine PM in A549 cells

Abstract

Background: Particulate matter (PM) has been associated to adverse health effects in exposed population and DNA damage has been extensively reported in *in vitro* systems exposed to fine PM (PM2.5). The ability to induce gene expression profile modulation, production of reactive oxygen species (ROS) and strand breaks to DNA molecules has been investigated in A549 cells exposed to winter and summer Milan PM2.5.

Results: A549 cells, exposed to 10μg/cm^2 of both winter and summer PM2.5,showed increased cytotoxicity at 24 h and a significant increase of ROS at 3 h of treatment. Despite these similar effects winter PM induced a higher number of gene modulation in comparison with summer PM. Both PMs modulated genes related to the *response to xenobiotic stimuli* (CYP1A1, CYP1B1, TIPARP, ALDH1A3, AHRR) and to the *cell-cell signalling* (GREM1) pathways with winter PM2.5 inducing higher fold increases. Moreover the winter fraction modulated also JUN *(cell-cell signaling),* GDF15, SIPA1L2 *(signal transduction*), *and* HMOX1 *(oxidative stress).*Two genes, *epiregulin* (EREG) and *FOS-like antigen1* (FOSL1), were significantly up-regulated by summer PM2.5. The results obtained with the microarray approach have been confirmed by qPCR and by the analysis of CYP1B1 expression. Comet assay evidenced that winter PM2.5 induced more DNA strand breaks than the summer one.

Conclusion: winter PM2.5 is able to induce gene expression alteration, ROS production and DNA damage. These effects are likely to be related to the CYP enzyme activation in response to the polycyclic aromatic hydrocarbons (PAHs) adsorbed on particle surface.

Keywords: PM2.5, gene expression, DNA damage, A549, ROS

1. Introduction

The Po Valley is a densely populated and industrialized area, and one of the most PM-polluted zones in Europe (Koelemeijer et al., 2006). According to emission inventory data for the Milan municipality, about 65% of the annual PM2.5 emissions derive from the traffic source, 20% from combustion for house heating and only the remaining 15% from industrial emissions, since heavy industries are no longer operating in the city area (Lombardy Region, 2007). Comparing the annual average of PM2.5 concentration found in this study and the previous ones (Lonati et al., 2008; Marcazzan et al., 2001) with the levels of other European countries (Querol et al., 2004), it results that annual PM2.5 levels in Milan (34 $\mu g/m^3$) are far higher than those observed at urban background sites in Northern and Southern Europe (8–15 $\mu g/m^3$ and 19–25 $\mu g/m^3$, respectively), and slightly higher than those observed in Central Europe (16–30 $\mu g/m^3$). On the other hand it has been reported that the PM2.5 levels in Milan are comparable to those of traffic exposed sites in Central and Southern Europe (22–39 μg/m3 and 28–35 μg/m3, respectively). Therefore, as PM2.5 chemical composition is similar to that of other European cities, relatively high PM2.5 concentration levels are peculiar of the Milan area.

The Milan PM2.5 fraction is dominated by combustion derived particles, consisting of a carbonaceous core with organic and inorganic compounds adsorbed on its surface (Sharma et al., 2007; Sevastyanova et al., 2008; Zerbi et al., 2008; Gualtieri et al., 2009) and its chemical characterization has received attention since 1997 (Marcazzan et al., 2001; Lonati et al., 2007, 2008).

The morphological damages produced on the human alveolar epithelial cells (A549) exposed to Milan winter PM2.5 have been previously reported

(Gualtieri et al., 2009) as well as the comparative pro-inflammatory potential of PM10 and PM2.5 on pulmonary cell lines (A549, BEAS-B2) (Gualtieri et al., 2010; Camatini et al., 2010). Moreover it has been recently demonstrated that the high cytotoxic and pro-inflammatory effects elicited by summer PM10 is partly related to its endotoxin content (Camatini et al., 2010). However recent data show that fine inhalable atmospheric particles are potential genotoxic agents (Billet et al., 2007, 2008; Sánchez-Pérez et al., 2009) for their ability to trigger oxidative stress leading to DNA damage (Risom et al., 2005). The fine PM fraction deserves thus particular attention due to its potential to trigger long term adverse effects.

Currently, the hypothesis that long-term exposure to air pollution can produce human morbidity and mortality is unanimously accepted and epidemiological evidences suggest an increased risk of lung cancer in people living in urban areas (Nerriere et al., 2005; Sen et al., 2007). Recent updates of the American Cancer Society cohort account for a strong association between ambient fine PM exposure and augmented risks of cardiopulmonary diseases and lung cancer mortality (Krewsky et al., 2005). Besides the experimental evidences demonstrating DNA damage in living systems after PM exposure, it has been shown that urban PM is able to induce germ cells mutation in exposed mice (Samet et al., 2004; Somers et al., 2004).

Among the chemical characteristics, which differ among PMs collected in different seasons, PAHs merit special attention. These compounds, present in almost all combustion-related emissions, have a well known genotoxic potential although other factors, such as metals, PM size, component interactions, and secondary chemical reactions may influence the PM genotoxicity (Binkova et al,. 1999; Topinka et al., 2000, Castano-Vinyals et al., 2004; Sevastyanova et al., 2008). PAHs require a metabolic activation by the CYP1 superfamily members to produce the reactive intermediates eliciting their adverse health effects on cell cultures (Billet et al., 2008). A

number of studies (Karlsson et al., 2006; Gutierrez-Castillo et al., 2006) have reported DNA damage, assessed by the comet assay, in the lung epithelial cell line A549 exposed to PM10 and PM2.5 sampled in different cities. The most significant result is that samples from urban sites, influenced by vehicle emissions, may cause a higher DNA damage compared to those sites with lower traffic emissions (Sharma et al., 2007).

The underlying mechanism in PM-related disease initiation is still largely unknown. Investigations addressing the effects of benzo[a]pyrene (BaP) on cellular gene expression patterns through microarrays are reported (Hockley et al 2006 and 2007, Kometani et al 2009) while no gene expression profiling has been analysed for the complex mixture of fine urban PM. Microarray technology allow querying the entire genome after exposure to an array of compounds, giving a characterization of the possible biological effects produced by such exposure (Gwinn et al., A. 2008). The identification of genes modified by the exposure to characterized PM2.5 exposure would provide not only a better understanding of the mechanisms responsible for its effects but also the identification of potential markers of exposure. The genome wide gene expression profiles and the molecular changes associated to urban PM, are here analysed on A549 cells treated with summer and winter PM2.5. The experiments were performed at concentration accordingly to the our previous data (Gualtieri et al., 2009; Gualtieri et al., 2010) and the data obtained related to the cell viability, intracellular reactive oxygen species (ROS) formation and DNA damage. Combining the classic toxicological approach with the toxicogenomic outputs, we report that urban fine PM may trigger genotoxic effects which might be mediated by transcriptional changes of cytochrome P450 enzymes, such as CYP1B1, as consequence of the concentration of PAHs adsorbed on fine PM.

2. Material and methods

2.1. PM collection and characterization

PM2.5 samples were collected daily, in summer (PM2.5 sum) and winter (PM2.5 win) season, in a representative urban site of Milan influenced by vehicular traffic. Samplers were located in a fenced area, about 20m from the nearest roads at about 2.5m from the ground, a height representative of exposure for typical populations. Low volume gravimetric samplers were used (EU system 38,33 l min-1, FAI Instruments, Rome; Italy).

PM2.5 samples were collected on Teflon (47mm Ø, 2µm, Pall Gelman, USA) and quartz filters (47mm Ø, 2µm, Whatman), the former being suitable to extract particles for biological investigations, the latter for chemical characterization. Before and after sampling, the filters were equilibrated for 48h (35% RH, at room temperature) and weighted with a M5P-000V001 microbalance (Sartorius, Germany) with a precision of 1µg.

The filters were then preserved in darkness at -4°C (to prevent photo-degradation and evaporation loss) until particle extraction or chemical characterization was performed.

Chemical characterization methods and results are available in Gualtieri et al. (2010) and Perrone et al. (2010). Briefly, the main parameter measured were water soluble inorganic ions, total carbon, PAHs and crustal and trace elements. Water soluble inorganic ions were analysed by ionic chromatography with the ICS-50 Ion Chromatography System (Dionex). Total carbon (TC) was measured by Thermal Optical Transmission (TOT) (Sunset Laboratory Inc., USA). Elements were investigated by an energy-dispersive type X-ray fluorescence analyzer (XRF, Spectra QuanX, Thermo Scientific), and the concentration of 14 elements were determined: Al, Si, K, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Br, Pb. For PAHs, filters were extracted in acetonitrile for 20 min in a ultrasonic bath, filtered to remove insoluble

fraction and analysed by HPLC-FD (Shimadzu, Kyoto, Japan). The chromatographic separation of PAHs was performed with a C18 column (Vydac PAH column, Alltech, USA, 150 Ø 4.6 mm I.D., particle diameter 5 lm and porosity 200A). Benzo[a]anthracene (B[a]A), chrysene (CHR), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[e]pyrene (B[e]P), benzo[a]pirene (B[a]P), indeno[1,2,3-c,d]pyrene (I[cd]P), benzo[ghi]perylene (B[ghi]P) were the PAH analysed.

2.2. Particle extraction

To obtain particles for *in vitro* exposures, Teflon filters were extracted using a Sonica® ultrasound bath by replicating four 20 minute cycles using 2ml of sterilized water for each filters' pool. Detached particles were then dried in a desiccator and suspended in sterilized water to obtain aliquots at a final concentration of 2µg/µl which were stored at -20°C until further use. Particles extracted from filters and resuspended for cell treatments were morphologically characterized by TEM analysis according to Gualtieri et al. (2009).

2.3. Cell culture and treatments

Human alveolar epithelial cells, A549 (American Type Culture collection), were routinely maintained in OptiMEM medium at pH 7.2, supplemented with 10% inactivated foetal bovine serum (FBS) and 1% penicillin/streptomycin and were grown at 37°C, with 5% CO_2. Cells were seeded at a concentration of $1.5x10^5$ in 12-well plate. After 24h from seeding, cells were treated with PM2.5 sum and PM2.5 win samples at the concentrations of 10µg/cm^2 in 1% FBS supplemented medium. PM2.5 exposures were extended for 24 h, with controls running parallel. Additional control groups were performed by exposing cells to carbon black particles (CB, nominal diameter < 2µm, Sigma Aldrich, Italy) at the same PM

concentration, to mimic particle-induced effect, and to benzo[a]pyrene (BaP) at a concentration of 14μM, as positive control for PAH-dependent metabolic activation.

2.4. Cell viability

MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to evaluate A549 cell viability after 24 h exposure to carbon black (CB), BaP, PM2.5 sum and PM2.5 win. At the end of the treatments, medium was discarded and cells were rinsed with PBS. MTT was added for 4 h at a final concentration of 0.3 mg/ml. The medium was removed and formazan crystals dissolved in DMSO. The absorbance of samples was measured by Multiskan Ascent (Thermo Scientific Inc.) at 570 nm.

To provide additional data on PM2.5 cytotoxicity, cell viability was evaluated also by fluorescence microscopy following cell staining with PI/Hoechst. Briefly, after 24 h of treatment, cells were washed in PBS, trypsinized, suspended in 500 μl of PBS supplemented with 20% of fetal calf serum and centrifuged at 250g for 10 min at 4°C. Supernatant was discarded and pellet suspended in 500 μl of medium supplemented with 20% of fetal calf serum and then mixed with the floating cell pellet collected at the end of cell treatment.

Cell suspensions were stained with Hoechst 33342/Propidium Iodide (Hoechst/PI) and smeared onto glass slide. Cells were scored (at least 300 cells per sample) according to the differential nuclear morphology and thus classified as 1) viable normal, 2) necrotic and 3) apoptotic cells, following the criterion previously described (Solhaug et al., 2004; Gualtieri et al., 2010).

The MTT and Hoechst/PI tests were replicated four and two times respectively and the final data were reported as the mean percent (± standard error of the mean, SEM).

2.5. ROS production

The quantitative measurement of intracellular ROS was investigated with flow cytometry. Twenty-four hours after seeding, A549 cells were incubated at 37° C with 5 μM 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in PBS for 30min and then treated with CB, BaP and PM2.5 win and sum for 3h. At the end of the exposure, cells were washed in PBS, trypsinized, pelleted and suspended in 500 μl of PBS. The ROS production after treatments, detectable by the oxidation of DCFH to dichlorofluorescein (DCF), was quantified by measuring the fluorescence intensity of 10,000 events with the cytometer EPICS XL-MCL (Beckman-Coulter) using 525 nm band pass filter. Data were analyzed using the EXPO32 ADC software (Beckman-Coulter). The experiment was replicated three times and the means (±S.D.) of fluorescence intensity were measured.

2.6. Genomic analyses

2.6.1. Total RNA extraction and purification

Twenty-four hours after seeding, A549 cells were treated with PM2.5 win and sum samples at the concentrations of 10μg/cm^2 in 1% FBS supplemented medium. PM2.5 exposures were extended for 24 h, with controls running in parallel. BaP treatment was also included. Three replicate samples were carried out for each condition and total RNA was extracted using the Qiazol reagent (Qiagen, Valencia, CA, USA), and purified using the miRNeasy total RNA Isolation Kit (Qiagen, Valencia, CA, USA). RNA integrity was verified by means of an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

2.6.2. Gene expression profiles

Biotin-labeled target were prepared from 1μg of total RNA using the GeneChip® Expression Analysis Technical Manual protocol (Affymetrix

Inc., Santa Clara, CA, USA). After fragmentation, 15 ug of cRNA obtained from PM treatment samples and from BaP treated samples were hybridized at 45° C for 16 hours onto GeneChip® Human U133 Plus 2 and Gene 1.0 ST arrays respectively. Following hybridization, non-specifically bound material was removed by washing and detection of specifically bound target was performed using the GeneChip® Hybridization, Wash and Stain kit, and the GeneChip® Fluidics Station 450 (Affymetrix). The arrays were scanned using the GeneChip® Scanner 3000 7G (Affymetrix) and raw data was extracted from the scanned images and analyzed with the Affymetrix Power Tools software package (Affymetrix).

2.6.3. Microarray and bioinformatic data analysis

The signals were converted to expression values using custom CDF (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp) and the BioConductor function for Robust Multi-array Analysis (RMA, Irizarry et al., 2003), in which perfect match intensities are background adjusted, quantile normalized, and log2 transformed. The quality control, filtering, statistical validation and data mining analyses were performed using the add-on BioConductor package OneChannelGUI. In particular, InterQuantile filtering and the non-parametric statistic test Rank Product method (p-value <0.05) were applied for filtering and identifying the differentially expressed genes (DEG), respectively. The DEG lists were annotated using the database for annotation, and integrated discovery DAVID bionformatics tool (http://david.abcc.ncifcrf.gov/; Huang et al, 2009) . The lists of genes modulated by PM2.5 win and sum exposure were investigated by Ingenuity Pathways Analysis (IPA, Ingenuity System, Montain view, Ca; USA). IPA is based on a database that integrates protein function, cellular localization, small molecule interaction and disease inter-relationship based on biomedical literature. The networks are displayed graphically as nodes,

representing individual gene/protein and edges representing the biological relation between nodes. Networks are ordered by score and optimized including as many differentially expressed genes as possible. A p-score for each possible network is computed. Therefore, networks with scores of 10 or higher have at least 99% confidence of not being generated by random chance alone. In the current study a score of 10 or higher was used to select highly significant biological networks.

2.6.4. Real time qPCR validation

Real time quantitative PCR (qPCR) analysis was performed by TaqMan assays (Applied Biosystem, Foster City, CA) on the following genes: CYP1A1 (Hs00153120_m1); CYP1B1 (Hs00164383_m1); TIPARP (Hs00296054_m1); GREM1 (Hs00171951_m1); CCL2 (Hs00234140_m1); GDF15 (Hs00171132_m1); EREG (Hs00914313_m1) INSL4 (Hs00171411_m1). Each test was carried out in triplicates according to standard protocol. For calculation of ΔCt GAPDH (Hs99999905_m1) was used as housekeeping gene. Data were calculated using the 2^ΔΔCt method comparing ΔCt of treated A549 cell to ΔCt control untreated samples. Reactions were incubated in Applied Biosystems 7900 Thermocycler. Ct values were calculated using the SDS software version 2.3 (Applied Biosystems) applying automatic baseline and threshold settings.

2.7. DNA damage

In order to evaluate DNA damage, strand breaks formation was analyzed by using the alkaline version of the Comet assay. Cells (1.5 $x10^5$) were seeded into 12-well culture plates and the day after were treated with CB (10µg/cm^2), BaP (14 uM) and PM2.5 win and sum (10 ug/cm^2). After 24 h cells were then trypsinized, centrifuged and suspended in 250µl PBS. Ten µl of cell suspension were mixed with 150µl of 0.5% low-melting point agarose

(Sigma-Aldrich), and 2 aliquots ($4.5 \cdot 10^3$ cells/slide) were added on a microscopic slide (Trevigen, USA).

After 1h in lysis buffer (NaOH 300mM, NaCl 2,5M, EDTA 100mM, Tris 10mM, DMSO 10% and Triton X-100 1%) at 4°C and 15min in DNA unwinding solution (NaOH 300mM, EDTA 1Mm) at RT, electrophoresis of the slides was run for 15min at 300mA, 25V at 4°C. Slides were washed in distilled water, dehydrated in 70% ethanol, stained with ethidium bromide (150μg/ml) and observed using a Zeiss Axioskop fluorescence microscope equipped with a Zeiss MRC5 digital camera. Three hundred nuclei for each sample were counted to estimate the percentage of cells with damaged DNA and 50 randomly selected nuclei to determine the tail moment using the dedicated software Casp (Comet Assay Software Project, University of Wroclaw, PL). The experiment was independently replicated four times.

2.8. CYP1A1 and CYP1B1 expression

Qualitative and quantitative measurements of CYP1A1 and 1B1 expression were evaluated both with cytofluorimetric and western blot techniques.

2.8.1. Immuno flow-cytometry

The quantitative protein expression of the cytochromes CYP1A1 and CYP1B1 was evaluated by flow cytometry. After a 24h treatment with 10 μg/cm^2 of winter and summer PM2.5, 10 μg/cm^2 of CB as control particles and 14 μM benzo[a]pyrene as positive control, cells were washed twice in PBS and trypsinized. Cells were then fixed in cold 1% paraformaldehyde for 15 minutes, pelletted at 1200 rpm for 7 min at 4°C, resuspended in 90% cold methanol and stored at -80°C for at least 24h. Once thawed, samples were washed once in PBS 0.5% BSA and incubated overnight with a goat polyclonal CYP1A1 or a rabbit polyclonal CYP1B1 antibodies (1:350 dilution, Santa Cruz) in PBS 0.5% BSA, 0.2% Triton X-100. Cells were then washed in PBS 0.5% BSA and incubated with an Alexa

Fluor 488-conjugated secondary antibody (1:500 dilution, Molecular Probes) in PBS 0.5% BSA, 0.2% Triton X-100, for 1 hour in the dark.

Finally, cells were washed once in PBS 0.5% BSA, resuspended in 500 µl PBS and analyzed on the Beckman Coulter EPICS XL-MCL flow cytometer. Fluorescence of 10.000 events was detected using 525 nm band pass filter.

2.8.2. Western blot

Cells were rinsed twice in PBS and protein extracts were obtained by solubilizing in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8;10% glycerol; 2% SDS; 5% b-mercaptoethanol) supplemented with protease inhibitors cocktail (20 µg/ml leupeptin hemisulpate, 10 µg/ml aprotinin, 20 µg/ml pepstatin, 50 µg/ml TPCK, 50 µg/ml TLCK and 1 mM PMSF), sheared through a 20 g syringe needle, and spun at 13000g for 30 min. Samples were clarified by centrifugation and the supernatant was boiled for 5 min. Western blots were performed according to Towbin et al. (1979). Briefly, proteins were quantified with Lowry method as modified by Peterson (1977) and 40 mg of total proteins for each sample were loaded onto a 10% SDS–PAGE gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Blots were washed twice in TBST (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.05% Triton-X100), blocked for 1 h at room temperature in TBS plus 5% (w/v) BSA, incubated overnight at 4 °C with different dilutions of monoclonal or polyclonal primary antibodies, washed twice in TBST and TBS (10 minutes each at room temperature), and incubated for 75 minutes at room temperature with a 1:3000 dilution of horseradish peroxidase-conjugated secondary antibody (Amersham) in TBS plus 5% BSA. Blots were finally rinsed once in TBS and three times in TBST and detected by enhanced chemiluminescent (ECL, Amersham Biosciences, Buckinghamshire, England) reaction and exposure to X-ray film. Primary antibodies included monoclonal anti-human CYP1A1 and polyclonal rabbit anti-human CYP1B1 (Santa Cruz Biotech) at dilutions of 1:200 and 1:170

respectively, polyclonal rabbit anti-human PARP7 and ALDH (ABCam) diluted 1:150. Monoclonal anti-beta-tubulin antibody (Sigma-Aldrich) was used as loading control.

2.9. Statistical analyses

For the cytotoxicity tests (MTT, ROS, Hoechst/PI, Comet) statistical differences between samples were tested with one-way ANOVA and post hoc comparisons performed with Dunnett's method, by using SigmaStat 3.1 software. Statistical differences were considered to be significant at the 95% level ($p < 0.05$).

3. Results

3.1. Cell toxicity

A significant reduction of cell viability measured by MTT assay after 24h of exposure was observed in cells exposed to PM2.5 win and PM2.5 sum, while cell viability was not significantly affected by CB and BaP (Fig. 1A). The cytotoxic effect of winter PM2.5 was confirmed by the Hoechst/PI scoring (Fig. 1B) which showed also an increase of apoptotic cells. PM2.5 sum induced mainly an increase of necrotic cells even though the cell viability reduction was not statistically significant.

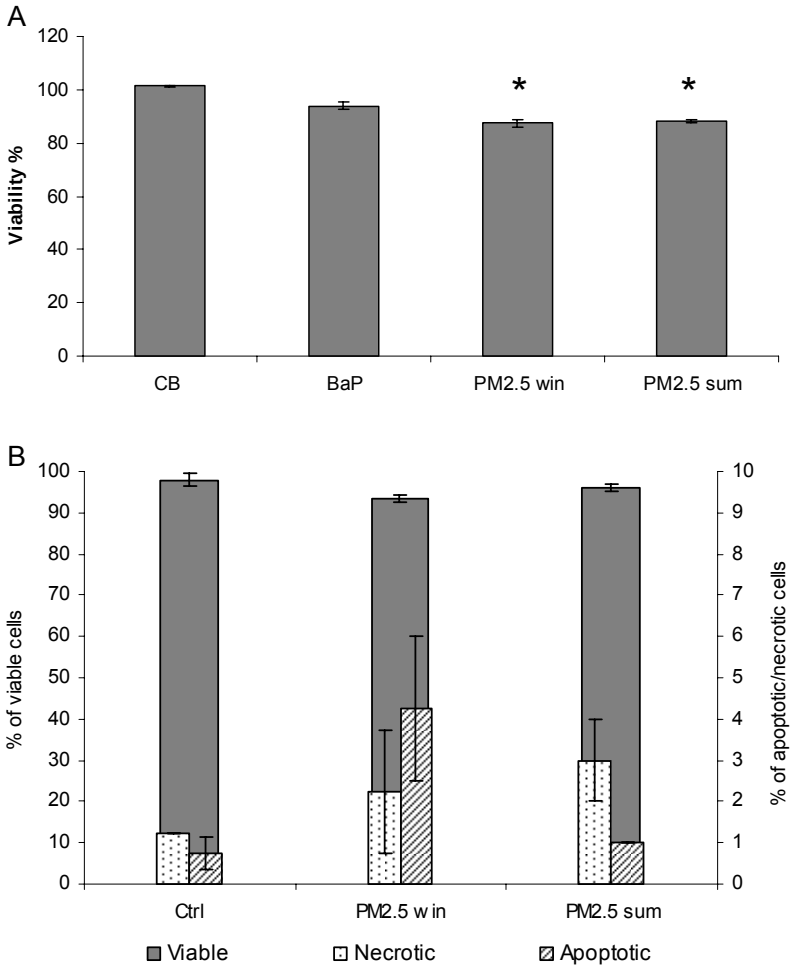

Figure 1. A) The histograms show A549 cell viability analysed with MTT assay, after cell exposure to Carbon Black (CB), PM2.5 win and sum at the dose of 10μg/cm2, and to benzo[a]pyrene (BaP) at 14 μM. The results are expressed as the mean (±S.E.M.) of percent decrease of viable cells in respect of control (100% viability) of four experiments in duplicate. B) Hoechst/PI scoring of A549 cells exposed to PM2.5 win and sum at the dose of 10μg/cm2 for 24h. Cells were scored as viable, necrotic or apoptotic accordingly to Gualtieri et al (2010). The results are expressed as the mean (±S.E.M.) * significantly different (One way ANOVA; $p < 0.05$) in comparison with control.

Since the formation of ROS is a early event in cells exposed to fine PM, their production was evaluated after 3 h of exposure by cytometric analyses with DCFH as fluorescent probe. ROS formation was significantly increased in cells exposed to PM2.5 win and sum (10 μg/cm^2) compared to control and BaP and CB exposed cells (Fig. 2).

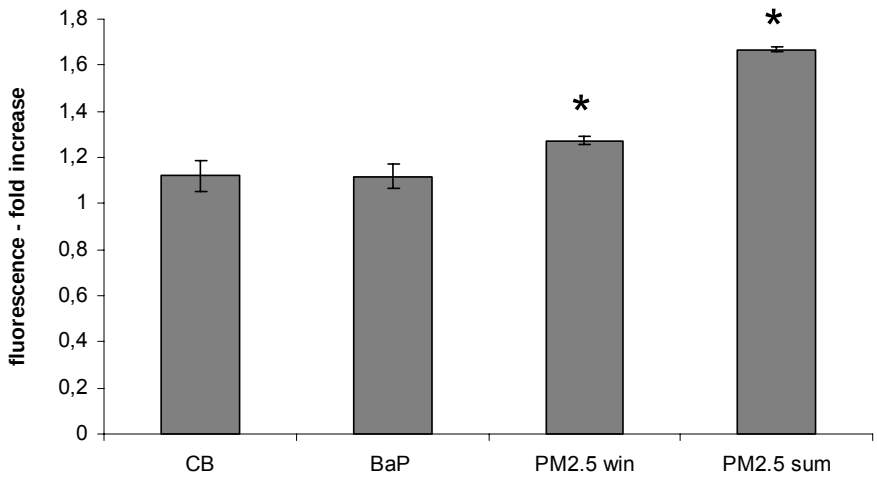

Figure 2. The histograms obtained by flow cytometry analyses show the mean fold increase (±S.E.M.) of fluorescence intensity, produced by ROS after cell exposure to 10μg/cm2 of Carbon Black (CB), PM2.5 win and sum and 14 μM benzo[a]pyrene (BaP). * significantly different (One Way ANOVA; $p < 0.05$) when compared to control.

3.2. Modulation of gene expression

Exposure of A549 cells to PM2.5 win and sum resulted in altered gene expression patterns in many RNA species. Global gene expression analysis revealed that 177 and 43 DEGs were modulated by PM2.5 win and sum respectively with p-value < 0.05 and log 2 fold change <-0.5 or > 0.5 (supplementary Tables 1 and 2). PM2.5 win induced the modulation of 177 genes, 68 were up-regulated and 109 were down-regulated. Among the up-

regulated genes, there were genes involved in the *oxidative stress* such as eme oxygenase 1 (HMOX1) and Jun oncogene (JUN) that is implicated in many signal transduction processes (Supplementary Table 1). Exposure of A549 to BaP significantly modulated 65 genes (22 up- and 43 down-regulated) among them *response to xenobiotic stimuli* such as CYP1A1, CYP1B1, TIPARP were the most up-regulated (Supplementary Table 3). Among the down regulated genes noteworthy of mention are CPS1, which encodes for the mithocondrial enzyme that catalyses the synthesis of carbamoyl phosphate, and PTGER2, which encodes a receptor for prostaglandin E2. Interestingly, the PM2.5 win and BaP shared the down-regulation of the E-cadherin gene (CDH1) associated to the epithelial mesenchymal transition (EMT). Treatment with PM2.5 sum triggered the modulation of a smaller number of genes: 16 up- and 26 down-regulated respectively. Interestingly, 29 DEGs were in common between the seasonal treatments and 13 displayed a similar pattern of expression (Table 1). In particular, winter treatment induced a relevant up-regulation of nine genes displaying a fold increase higher than 1.5 (log2FC of 0.75), six of which were up-regulated also by PM2.5 sum, even though at a lower extent (CYP1A1, CYP1B1, TIPARP, ALDH1A3, AHRR, GREM1, Table 1). Two genes, epiregulin (EREG) and FOS-like antigen1 (FOSL1), were up-regulated only by PM2.5 sum. The DEGs were classified according to their function. Winter fine PM modulated many genes involved in *response to xenobiotic stimuli, cell-cell signaling and signal transduction, nucleic acid metabolic process, inflammatory response*.

Table 1: List of differentially expressed gene modulated in A549 cells treated with summer and winter PM2.5. Data are shown as FoldChange (FC). Red, up-regulated DEG; Green, down-regulated DEG; White, not regulated)

Table 1 List of the common differentially expressed genes (DEG)

Entrez Gene	Gene Symbol	Description	Cellular Localization	Functional Classification	PM2.5 win	PM2.5 sum
1543	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	intracellular	response to xenobiotic stimulus	⇧3.59	⇧0.86
1545	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	intracellular, membrane	response to organic substance	⇧3.24	⇧1.95
25976	TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	intracellular	protein metabolic process	⇧2.77	⇧1.33
220	ALDH1A3	aldehyde dehydrogenase 1 family, member A3	intracellular, cytoplasm	alcohol metabolic process, vitamin	⇧1.89	⇧0.86
57491	AHRR	aryl-hydrocarbon receptor repressor	intracellular	nucleobase, nucleoside, nucleotide	⇧1.89	⇧1.03
26585	GREM1	gremlin 1, cysteine knot superfamily	extracellular	cell-cell signaling	⇧1.83	⇧1.79
6347	CCL2	chemokine (C-C motif) ligand 2	extracellular	inflammatory response	⇧1.40	⇨0.61
57568	SIPA1L2	signal-induced proliferation-associated 1 like 2	intracellular	signal transduction	⇧1.07	⇨0.48
9518	GDF15	growth differentiation factor 15	extracellular	signal transduction,cell-cell signaling,	⇧1.00	⇨0.27
8870	IER3	immediate early response 3	membrane	apoptosis	⇧0.98	⇨0.59
6775	STAT4	signal transducer and activator of transcription 4	intracellular, cytoplasm,	nucleobase, nucleoside, nucleotide	⇧0.83	⇨0.55
2069	EREG	epiregulin	extracellular	regulation of progression through	⇨0.69	⇧1.10
8061	FOSL1	FOS-like antigen 1	intracellular	nucleobase, nucleoside, nucleotide	⇨0.47	⇧1.16
6857	SYT1	synaptotagmin I	intracellular, cytoplasm	cell-cell signaling	⇨-0.14	⇩-0.76
1E+05	CPNE4	copine IV			⇩-0.75	⇨-0.46
182	JAG1	jagged 1	plasma membrane	cell motility	⇩-0.75	⇨-0.23
57210	SLC45A4	solute carrier family 45, member 4			⇩-0.76	⇨-0.25
2266	FGG	fibrinogen gamma chain	extracellular	protein metabolic process	⇩-0.78	⇨-0.50
727	C5	complement component 5	extracellular	inflammatory response	⇩-0.79	⇨-0.35
2E+05	PRICKLE 2	prickle homolog 2	intracellular	establishment and/or maintenance of	⇩-0.80	⇨-0.17
10891	PPARGC 1A	peroxisome proliferator-activated receptor gamma,	intracellular	protein metabolic process	⇩-0.85	⇨-0.20
22943	DKK1	dickkopf homolog 1	plasma membrane	regulation of biological process	⇩-0.86	⇨-0.34
4884	NPTX1	neuronal pentraxin I	intracellular, cytoplasm	cell-cell signaling	⇩-0.86	⇨-0.56
7494	XBP1	X-box binding protein 1	intracellular	nucleobase, nucleoside, nucleotide	⇩-0.86	⇨-0.42
5274	SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member		system development	⇩-0.87	⇩-1.02
80323	CCDC68	coiled-coil domain containing 68			⇩-0.88	⇨-0.40
2114	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	intracellular	nucleobase, nucleoside, nucleotide	⇩-0.89	⇨-0.35
6374	CXCL5	chemokine (C-X-C motif) ligand 5	extracellular	inflammatory response	⇩-0.90	⇩-0.95
10468	FST	follistatin		nucleobase, nucleoside, nucleotide	⇩-0.90	⇨-0.30
654	BMP6	bone morphogenetic protein 6	extracellular	nucleobase, nucleoside, nucleotide	⇩-0.92	⇨-0.43
4929	NR4A2	nuclear receptor subfamily 4, group A, member 2	intracellular	nucleobase, nucleoside, nucleotide	⇩-0.93	⇨-0.62
3399	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-	intracellular organelle	nucleobase, nucleoside, nucleotide	⇩-0.94	⇨-0.41
5732	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	plasma membrane	signal transduction	⇩-0.99	⇨-0.65
7103	TSPAN8	tetraspanin 8	intracellular, cytoplasm	protein metabolic process	⇩-0.99	⇨-0.39
84620	ST6GAL2	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2	intracellular	protein metabolic process	⇩-1.00	⇨-0.42
6446	SGK1	serum/glucocorticoid regulated kinase 1	intracellular	metal ion transport, apoptosis	⇩-1.02	⇩-0.76
1373	CPS1	carbamoyl-phosphate synthetase 1, mitochondrial	intracellular, cytoplasm	nucleobase, nucleoside, nucleotide	⇩-1.03	⇨-0.50
56521	DNAJC12	DnaJ (Hsp40) homolog, subfamily C, member 12	membrane	protein metabolic process	⇩-1.05	⇨-0.74
2861	GPR37	G protein-coupled receptor 37 (endothelin receptor type B-	intracellular, nuclear	signal transduction	⇩-1.07	⇩-0.87
2E+05	RHOV	ras homolog gene family, member V	intracellular, membrane	signal transduction	⇩-1.13	⇩-0.85
6616	SNAP25	synaptosomal-associated protein, 25kDa	plasma membrane	cell-cell signaling	⇩-1.17	⇩-0.83
3641	INSL4	insulin-like 4	extracellular	cell-cell signaling	⇩-1.36	⇨-0.73
3400	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-	intracellular, organelle membrane	nucleobase, nucleoside, nucleotide	⇩-1.48	⇨-0.42
6662	SOX9	SRY (sex determining region Y)-box 9	intracellular	nucleobase, nucleoside, nucleotide	⇩-1.77	⇩-1.29

Genes up regualated (red, ⇧) and downregulated (green, ⇩), in cells exposed to 10μg/cm^2 summer and winter PM2.5. Data are shown as Log2Fold Change (FC).

Using IPA analysis for the functional annotation of DEGs lists, four relevant networks with score higher than 10 were defined (Table 2).

Table 2 List of the gene expressed in A549 cells exposed to10μg/cm2 summer and winter PM2.5 in their networks and main functions.

Gene in the networks	Score	Focus Molecules	Top Functions
PM 2.5 win			
Ahr-Arnt, Ahr-aryl hydrocarbon-Arnt, AHRR, ALDH1A3, ALP, BMP6, C5, C13ORF15, CCL2, CHEMOKINE, Creb, CXCL5, CYP1A1, CYP1B1, ETS2, FGG, FST, GDF15, GREM1, hCG, ID3, IER3, IL1, IL12 (complex), Il12 (family), LDL, NFkB (complex), NR4A2, P38 MAPK, PDGF BB, PPARGC1A, PTGER2, SOX9, STAT4, Tgf beta	53	21	Xenobiotics Metabolism, Inflammation, Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
Akt, DKK1, DNAJC5, DOT1L, ENaC, ERK, ERK1/2, estriol, FSH, GDF15, HDC, Histone h3, HMGN1, ID4, IL17F, IL28A, IL8RA, INSL4, Insulin, MFN2, MIR122, OLIG2, PI3K, PIKFYVE, PPP1R13L, PPP2R5B, PRICKLE2, RNA polymerase II, SERPINI1, SGK1, SNAP25, SYT9, TAC1, TIE1, VAMP8	16	8	Behavior, Nervous System Development and Function, Cellular Movement
PM 2.5 sum			
ALDH1A3, APP, AXIN2, CBR3, CXCL5, EREG, FOSL1, FSH, GANAB, GPR37, GREM1, H19, Hsp70, KCNB1, methylamine, NFkB (complex), PDGF BB, PRSS2 (includes EG:25052), RHOV, SERPINI1, SGK1, SLC14A2, SLC9A1, SLIT2, SNAP25, Snare, STX2, STX1B, SV2A, SYT1, SYT2, SYT3, UNC13B, WNK1, ZDHHC17	31	12	Cell Signaling, Cellular Assembly and Organization, Cellular Function and Maintenance
2-hydroxyestradiol, 2-methoxyestradiol, 7-ketocholesterol, AHR, Ahr-Arnt, Ahr-aryl hydrocarbon-Arnt, AHRR, AIP, ALDH, AMH, ARNT, CYP1A1, CYP1B1, Cytochrome p450, estriol, FGF10, KLF2, lipoxin A4, LOC729505, melatonin, MUC2, NQO1, PARP, POR, SOX9, TIPARP, TNF, TRIP11, TXN, TYR, UGT1A6, vitamin A	11	5	Gene Expression, Endocrine System Development and Function, Small Molecule Biochemistry

Table 2: Significant networks identified by the IPA analysis on the list of differentially expressed genes. The score column indicates the -log(p-value), while the focus molecules column quantifies the number of modulated genes in the network.

The top functions of the main IPA networks were those of *Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism* and *Cell signaling, cellular assembly and organization, cellular function and maintenance* PM2.5 win and PM2.5 sum modulated genes respectively (Table 2). In particular, PM win treatment was associated with a large network (score =53) involving 21 genes including significant genes, such as NF-kB and p38 MAPK, known to be related with CYP1A1, AHRR, CYP1B1, GREM1, HMOX1 and STAT4 genes (Fig 3).

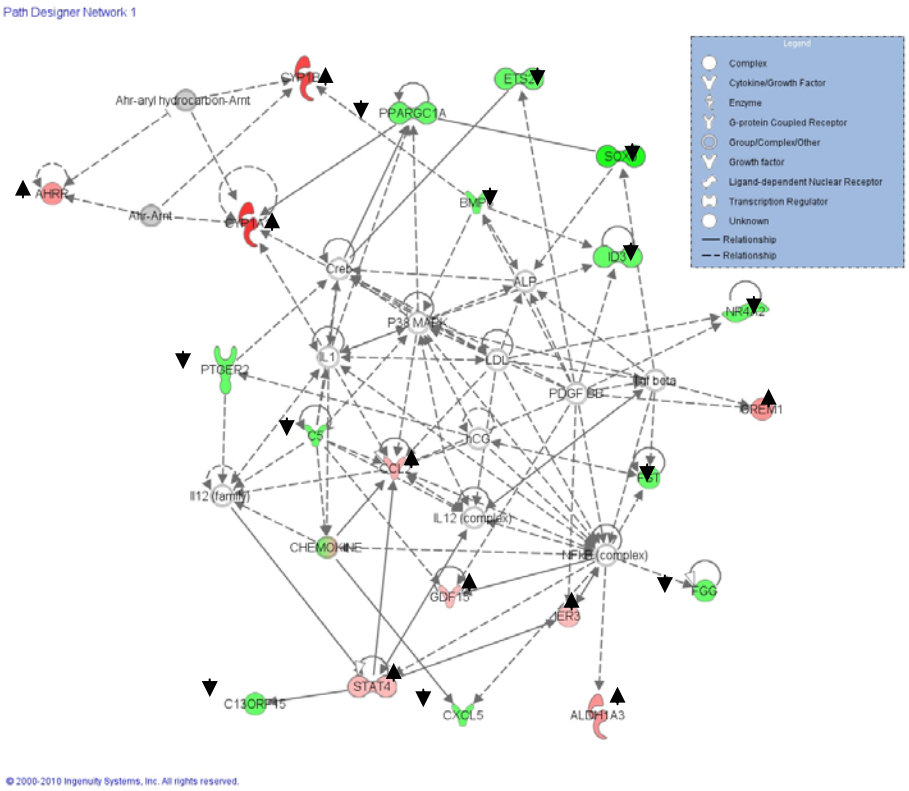

Figure 3. Signalling network of genes modulated in cells exposed to winter PM2.5. Green and red colours are associated to down-modulated and up-regulated genes respectively (see also supplementary Table 1). Dashed lines indicate indirect interaction between genes.

Validation by qPCR analysis on CYP1A1, CYP1B1, TIPARP, GREM1, CCL2, GDF15, EREG, INSL4 genes confirmed microarray data (Table 3). In particular an up-regulation of CYPs and TIPARP genes after PM2.5 winter treatment was evident while EREG was more up-regulate by summer PM.

Overall fine PM induced a relevant modulation of several genes involved in the metabolism of PAHs (i.e. BaP), which were further analyzed for validation at protein levels in cells treated also with positive (BaP) and negative (CB) inducers (see below).

Table 3. qPCR data validation, data are expressed as 2^ΔΔCt

qPCR analysis (2^ΔΔCt)		
gene symbol	*PM2.5 sum*	*PM2.5 win*
CYP1A1	5.23	53.64
CYP1B1	7.26	11.57
TIPARP	3.17	9.30
GREM1	4.07	4.21
CCL2	1.63	2.52
GDF15	1.34	1.87
EREG	2.93	1.34
INSL4	-1.66	-3.63

Table 3: Validation of microarray data by real time qRT-PCR analysis. Results are expressed as 2^ΔΔCt.

3.3. DNA damage

DNA damage, measured as strand breaks by alkaline Comet assay, was evident mainly after PM2.5 win exposure (Fig. 4). BaP, a recognized pro-carcinogenic PAH, induced a very high DNA damage level (Fig. 4), while CB did not. Compared to PM2.5 sum, PM2.5 win resulted more harmful in inducing both higher number of DNA damaged cells and higher amount of

DNA lesions per cell, as evidenced by the significant increase in the tail moment values (Fig. 4).

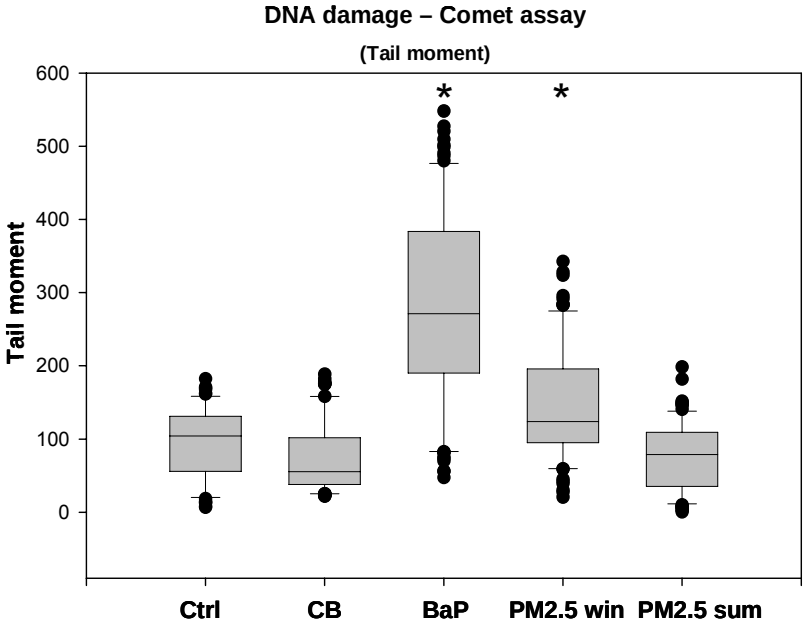

Figure 4. DNA damage evidenced with Comet assay in cells exposed to 10μg/cm^2 of Carbon Black (CB), PM2.5 win and sum and to 14 μM benzo[a]pyrene (BaP). Box plot illustrates the comet tail moment. * significantly different (One Way ANOVA; $p < 0.05$) when compared to control.

3.4. CYPs expression

Validation of the GEP data was performed by protein expression analysis of the most significant up-regulated genes, such as CYPs, TIPARP and ALDH1A3. At the condition used, neither immune cytometric technique, nor western blotting were able to detect differential expression of CYP1A1, TIPARP and ALDH1A3 (*data not shown*) in A549 cells exposed to PM2.5. Also BaP failed to induce detectable levels of such proteins. However the levels of CYP1B1 expression strongly supported the results obtained with GEP and closely reflected the genotoxic activity produced by PM2.5 winter, which induced a significantly higher CYP1B1 synthesis than in controls.

PM2.5 sum and CB did not, showing protein levels comparable to those of controls (Fig. 5). As expected, BaP worked as positive CYP1B1 inducer, as previously observed for DNA damage. These findings were consistent and supported by both cytofluorimetric and western blotting analyses (Fig 5).

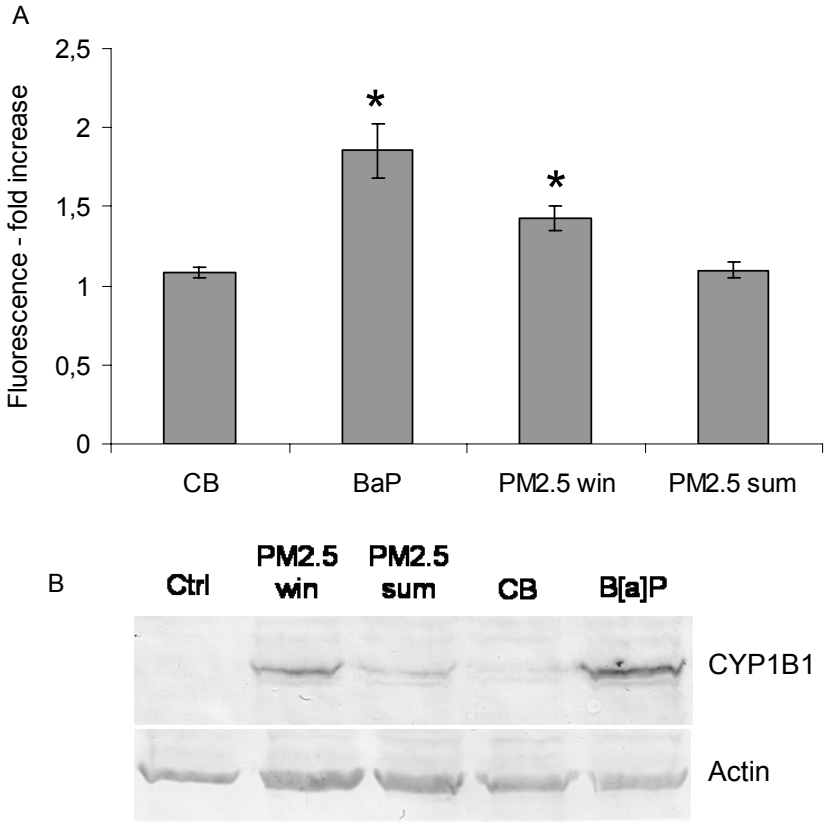

Figure 5. A) The histograms, show the CYPB1 expression (based on flow cytometry analysis) in cells exposed to 10μg/cm^2 of Carbon Black (CB), PM2.5 win and sum and to 14 μM of benzo[a]pyrene (BaP). The mean (±S.E.M.) fold increase of fluorescence intensity to the respect of control (fold increase=1) of three experiments is presented (*) significant difference (One Way ANOVA; $p < 0.05$) when compared to control. B) Western blot representative of the CYP1B1 expression in A549 cells exposed to 10 μg/cm^2 of Carbon Black (CB), PM2.5 win and sum and to 14 μM of benzo[a]pyrene (BaP) for 24 h.

4. Discussion

A correlation between PM and human health risk has been described in epidemiological studies on US population cohorts indicating a statistic-based relation between lung cancer and human exposure to high levels of urban fine PM (Zanobetti and Schwartz, 2009; Pope et al, 2009; Krewski et al, 2005). Besides these epidemiological evidences, laboratory data on the ability of PM to induce tumorigenic effects are rather scanty. However several experimental data obtained on *in vitro* and *in vivo* systems suggest that the fine PM, principally originated from combustion processes, can induce genotoxic, mutagenic and non genotoxic pathways responsible of pro-carcinogenic mechanisms (Andrysik et al., 2011; Bonetta et al. 2009). The data here presented are focused on the effects triggered by the fine PM collected in a city with a dense vehicular traffic aiming to improve the knowledge on the molecular pathways involved in the genotoxic and mutagenic pathways activated by such particles and to eventually identify the chemical elements responsible of such event.

PM10 and PM2.5 levels in Milan, as previously reported, are over the limits established by the EU. Numerous literature data outline the exacerbation of respiratory and cardio-circulatory diseases in human population chronically exposed to PM (Englert, 2004; Pope et al. 2009). Recent studies, performed on *in vitro* and *in vivo* systems exposed to PM, have outlined that the most severe inflammatory effects are mediated by the summer coarse PM fraction (PM10) (Camatini et al., 2010; Becker et al 2005; Hetland et al 2004, Schins et al 2002) characterised by a high endotoxins content which induces pro-inflammatory response and necrotic cell death in pulmonary cultured cells (Camatini et al., 2010; Gualtieri et al., 2010) and in mice lungs (Farina et al., 2011). On the other hand fine PMs and fine PMs organic extracts mainly induced DNA damages

(Sevastyanova,et al., 2008; Andrysik et al., 2011). The role of PM-induced DNA damage has been largely debated also in relation with cell cycle arrest, programmed cell death (Dagher et al., 2006; Gualtieri et al., 2011) and the capability of fine PM to inhibit DNA repair mechanisms (Mehta et al., 2008). Given these data the PM activation of pro-carcinogenic gene pathways needs further investigation.

GEP analysis on A549 cells exposed to summer and winter PM2.5 demonstrated that PM2.5 win is the most efficient gene modulator, both in term of number of modulated genes and of the intensity of modulation. Interestingly, functional analysis of genes expression revealed that PM2.5 win majorly contributed to the modulation of genes implicated in transcription regulation and cancer development, such as CYP1A1 and CYP1B1. As previously reported (Gualtieri et al., 2010; Perrone et al., 2010), it is well known that Milan PM2.5 win has a higher quantity of PAHs. The sum of the eight major PAHs analysed in PM2.5 was ten times higher in winter samples (0.031% by mass) in comparison with the summer ones (0.003% by mass). Thus the hypothesis assumed is that the organic components adsorbed to fine particles are the determinant of the biological effects observed. Since BaP is known to be a reference of pro-carcinogenic PAH, it was here used as positive control for PAH-induced effects, and for comparing the effects produced by BaP and PM2.5. As shown, this parallelism was positively maintained for both the genotoxicity and GEP. BaP resulted to be a potent DNA damaging (data from Comet Assay) and a gene modulator agent (*supplementary table 3*). Indeed the results obtained showed similar modulation of CYP1A1, CYP1B1, TIPARP, AHRR, ALDH1A3, GDF15 genes by PM2.5 win and BaP treatments. Consistently with our data, similar results has been reported on MCF7 cell lines (Hockley et al., 2006) while the up-regulation of GDF15 and JUN genes was reported in HepG2 cells exposed at low dose of BaP (2.5 μM) (Hockely et al., 2007).

Moreover, the PM2.5 win and BaP exposure down-regulated of E-cadherin gene (CDH1), which has been associated to epithelial mesenchymal transformation (Yoshino et al., 2007). These data suggest that the increased amount of PAHs may be related to the increased modulation of the genes involved in signal transduction, apoptosis, metabolic activation process and *cell – cell signalling* events, which are present in several pathological conditions including cancer. This conclusion is supported also by the paper of Castorena-Torres et al. (2008) who describe a significant increase of CYP1A1 and CYP1B1 genes in A549 and HepG2 cells exposed to PAHs extracted from soil samples collected near a coke oven factory.

PAHs are widely distributed in the environment (water, soil and air) and usually derive by an incomplete combustion process from engine exhaust, cigarette smoke and wood fire. In urban PM, PAHs are commonly associated to ultrafine particles. A prolonged exposure to low levels of complex PAHs' mixture poses a serious problem due to an increase of risk for human health adverse effects. PM mixtures have the ability to induce CYP1A1 and CYP1B1 and contribute to DNA binding, as shown by Mahadevan et al. (2004), who have analysed a PAH mixture, simulating the PAH content of a standard diesel exhaust particle (DEP), of urban atmospheric PM and of coal tar on MCF-7 cells. They found a significant induction of CYP1A1 and CYP1B1, confirming the carcinogenic potential of the PAHs present in such mixtures. While the individual PAHs carcinogenicity mainly depends on the structure of the bioactivated molecule, the extent of the bioactivation is a direct function of the available CYP enzymes, which in turn are dependent on the protein synthesis induction. The CYP induction, produced by Milan winter PM2.5 is very similar to the one produced by BaP, even at a lesser extent and it thus may be a marker of the fine winter PM exposure. Although the protein expression has been confirmed only for the CYP1B1, in BaP and winter PM2.5 exposed cells, possibly due to the low doses used and/or to

specific pathways of the A549 cell line, the data are consistent with previous results (Gualtieri et al., 2011). Moreover the activation of HMOX1 gene by winter PM confirms the importance of oxidative stress related responses and the quinones present in winter PM may be responsible of the ROS increase here reported. Nakayama Wong et al (2011) have recently reported a similar pattern of gene expression for both Cyp and oxidative stress related genes in PMs with high concentrations of PAHs thus confirming the importance of the organic compounds in triggering xenobiotics and oxidative stress genes expression.

The expression of CYP1B1 is usually induced by hormones and ligands of the aryl hydrocarbon receptor (AhR). The data available suggest the activation of the AhR by PM components which may act as a dioxin-like molecules (Arrieta et al 2003, Wenger et al 2009, Ferecatu et al 2010), thus increasing Cyp genes expression. The activation of the AhR has been associated also to non-genotoxic pro-carcinogen molecules, however our data suggest a major activation of the receptor in response to the presence of PAHs.

Winter PM2.5 induced the activation of the TIPARP gene, which has been directly related to the activity of TCDD in cell culture (Diani-Moore et al 2010, Ma 2002). The up-regulation of this gene in PM-treated cells suggests that this class of compounds may be adsorbed onto particles and thus induce tumour promotion through non-genotoxic pathways. Till now data on the presence of dioxins associated with PM are scanty. Recently Olivares et al. (2011) have reported that most of the PM dioxin-like activity should be related to PAHs or to other polycyclic aromatic compounds (ketones or quinones). Our results in part confirm this hypothesis, since summer PM2.5, with low level of PAHs, induces a not significant activation of CYP enzymes and TIPARP genes. The role of genotoxic effects over the non genotoxic ones in winter PM2.5 is confirmed also by the down-regulation of the E-

cadherin gene (CDH1). Winter PM2.5 down-regulated also CPS1 and PTGER2 genes. Their down-regulation has been related to DNA methylation (Liu et al., 2011; Tian et al., 2008) and the development of tumors in hepatic and lung tissues. It is thus tempting to speculate that winter fine PM triggers epigenetic modification in exposed cells by promoting DNA hyper-methylation. Accordingly Pavanello et al. (2009) reported that the chronic exposure to PAHs enriched PM induced significant higher DNA methylation in workers at high lung cancer risk. Our data thus suggest that the levels of PAHs adsorbed on fine PM may have an important role on DNA methylation inducing epigenetic alterations.

Noteworthy of mention is the GREM1 gene up-regulation similarly triggered by summer and winter PM2.5. This gene has been shown to be up-regulated in lung in response to hypoxia (Costello et al., 2008) and also in lung tissues from patient with pulmonary fibrosis (Myllarniemi et al., 2008). It will be possible to deduce that, for its presence in both PM, GREM1 gene is up-regulated in response to the physical properties of the particles rather than their chemical components. If this may be the case, it can be speculated that the biological effects may be initially evoked by the only presence of fine particles on the alveolar epithelium.

Of interest is also the ability of summer PM2.5 to up-regulate the FOSL1 gene in addition to JUN. Indeed the Fos gene family, constituted of 4 members (FOS, FOSB, FOSL1, and FOSL2) encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1, implicated in the regulation of cell proliferation, differentiation, and transformation. These data, although to be confirmed, suggest that summer PMs may activate proto-oncogenes in response to non genotoxic stimuli (Andrysik et al 2011), However this hypothesis need further analyses since the data here reported for the summer

PM showed a decrease of cell proliferation/viability (MTT assay) rather than an induction of the proliferation, as reported by Andrysik et al (2011).
In conclusion cytotoxicity and DNA damage are the most evident effects produced by winter Milan PM2.5 on A549 cells. The gene expression profiling has evidenced that cytochrome P450 family genes together with AHRR,TIPARP and ALDH1A3 are consistently induced by fine PM and winter PM is more potent in the induction of many pro-carcinogenic genes (i.e. JUN, FOS). Although the validation at protein levels has not been obtained and there is a lack of correlation with the gene expression data, a panel of genes including CYP1A1 and CYP1B1, which need further investigation, are here proposed. The data presented underline also the possible role of non genotoxic mechanisms in PM2.5 induced effects and the ability of winter fine PM to alter DNA methylation. Additional analysis of gene and protein expression in different human pulmonary cell lines will allow confirming these results, which outline the significant role of the PM chemical composition in triggering the biological effects.

Conflict of interest

None to be declared.

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Author Contributions

MG and EL performed the analyses on particulate matter treated A549 cell lines and carried out data validation by western blotting. MM performed the microarray experiments and applied computational methods for the analysis of microarray data. EM, VT applied IPA computational methods for the analysis of microarray data. VT and MP carried out qPCR analysis. CB assisted with the design and interpretation of experiments. MG and CB wrote the manuscript. All authors read and approved the final version of the manuscript.

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Paper IV

Winter PM2.5 induces a mitotic delay which is caused by components in the organic fraction

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Running title: Organic fraction drives winter PM2.5-induced mitotic delay

Abstract

We have previously shown that exposure of human epithelial BEAS-2B cells to winter PM2.5 from Milan may cause mitotic arrest resulting in mitotic-apoptosis. Here we more specifically explore the mechanisms involved in PM-induced mitotic delay and elucidate particle properties responsible of these effects. A lower dose of winter PM2.5 induced an early G2 arrest (after 3 h) which was followed by an arrest in metaphase/anaphase (M/A) transition point. Examination under fluorescence microscopy revealed the presence of mitotic spindle aberrations. This arrest was followed by a mitotic slippage that resulted in tetraploid G1 cells and cells with micronuclei. Further analysis of winter PM2.5-related cellular effect showed that PM induced reactive oxygen species (ROS), through mitochondrial damage as well other pathways. Fractionation of PM into organic fraction and washed particles suggested that the G2/M arrest as well as the formation of ROS were due to chemicals in the organic fraction. Lastly, winter PM2.5 was shown to cause DNA damage through the formation of reactive electrophilic/radical metabolites via a CYP P450 dependent reaction.

Key words: PM2.5, BEAS-2B, mitotic arrest, CYP enzymes, ROS

1. Introduction

Particulate matter (PM) is a notorious air pollutant whose adverse effects on human health are well established (de Kok et al., 2006; Englert, 2004). Increased levels of PM have been associated with exacerbation of airways disease in patients with asthma and Chronic Obstructive Pulmonary Disease (COPD) (Donaldson et al., 2003). Growing evidence has linked long-term exposure to fine PM (PM2.5; aerodynamic diameter ≤ 2.5 μm) with increases in the risk of cardiovascular mortality (Dockery et al., 1993; Pope and Dockery, 2006; Brook et al., 2010; Pope et al., 2011) and partly also to lung cancer (Pope et al., 2002; Pope et al., 2011). However, the understanding of the mechanisms by which PM exerts its various adverse effects is still incomplete and detailed *in vitro* studies are highly needed. The difficulty of this topic partly lies in the variability and complexity of this compound's structure and composition.

Urban air PM is a heterogeneous mixture of various types of particles originating from different sources. Combustion particles emitted from vehicles consist mainly of spherical primary carbon particles with diameters ranging from 20 to 30 nm, which tend to aggregate (PM1 and PM2.5; Gualtieri et al., 2009; Kocbach et al., 2005). The small diameters of the primary carbon particles thus provide a relatively large surface area per mass unit, that facilitates the adsorption of various components to the particle surface, including metals, organic compounds, allergens and bacterial components like endotoxins (Alfaro-Moreno et al., 2002; Soukup and Becker, 2001). In contrast particles of larger size (PM10) more often are found to be arbitrarily-shaped mineral particles from road wear and soil dusts (Chung et al., 2008). Composition of urban air PM also varies with season, and all of these variables are of fundamental importance when determining their biological effects. This is evidenced by *in vitro* studies

showing that, depending on composition, PM can trigger release of inflammatory mediators including various cytokines and chemokines (Hetland et al., 2004; Alfaro-Moreno et al., 2002), genotoxic effects (Billet et al., 2008; de Kok et al., 2005; Don Porto Carero et al., 2001) and cell death (Alfaro-Moreno et al., 2002; Hsiao et al., 2000).

In vitro studies have found that PM reduces cell growth, not only by causing cell death, but also as a result of reduced proliferation (Poma et al., 2006; Kocbach et al., 2008; Gualtieri et al., 2011a). This has been linked to various alterations in cell cycle (Deng et al., 2007; Kocbach et al., 2008; Jalava et al., 2007; Gualtieri et al., 2011a).

Cell cycle progression can be blocked or delayed in response to genotoxic stresses or structural dysfunctions. Induction of DNA damage is considered to be one of the main causes for cycle arrest and DNA-integrity checkpoints including G1/S, G2/M and metaphase-anaphase (M/A) transitions, activate pathways which determine the delay of the cell cycle (Rieder, 2011; Wang et al., 2009; Okada and Mak, 2004). Generally, the protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) detect the DNA damage and give rise to signals that, through the activation of checkpoint proteins such as p53, Chk1 and Chk2 kinases, results in cell cycle delay and/or cell death. The cell cycle is regulated by G1/S or G2/M transition promoters like cyclin-Cdk (Pearce and Humphrey, 2001; Branzei and Foiani, 2008). Cyclin B1, in association with the cyclin-dependent kinase 1 (Cdk1), drives the progression from G2 to mitotic phase. Once into mitosis, the passage from metaphase into anaphase (M/A transition point) requires the destruction of the Cdk1/cyclin B1 complex. The anaphase-promoting complex (APC) is responsible for the ubiquitination and subsequent degradation of cyclin B (Castedo et al., 2004).

The spindle assembly checkpoint (SAC) functions to delay mitosis, at the M/A transition point, preventing the activation of APC until the mitotic

spindle is correctly formed (Pearce and Humphrey, 2001; Fukasawa, 2008). The inhibition of APC by SAC results in the stabilization of cyclin B, and this prevents the anaphase onset and nuclear division until all chromosomes are properly attached to the bipolar mitotic spindle (McNeely, 2008; Castedo et al., 2004). If the spindle is not properly attached to chromosomes in a certain time period, the cell can enter a death process or can exit from mitosis without divide the genetic material, a process named mitotic slippage. Cell death during mitosis or after mitotic slippage is often termed mitotic catastrophe, an atypical mode of cell death which is due to premature or inappropriate entry into mitosis (Castedo et al., 2004). Abnormal spindle structure can be a later effect of DNA damage or can be directly originated by spindle-poisons. Thus whether a particular agent inhibits progression through G2/M or M/A transition point is of great importance in the understanding of mechanism of action involved (Rieder, 2011).

In a recent study we reported that Milan winter-PM2.5 could cause mitotic arrest and mitotic catastrophe in the human lung epithelial cell line BEAS-2B (Gualtieri et al., 2011a). The aim of the present study was to more specifically explore mechanisms involved in Milan winter PM-induced mitotic delay and to elucidate particle properties involved in these effects.

2. Materials and Methods

2.1. PM collection and preparation

PM samples were collected during the winter and summer season (2010) in Torre Sarca, an urban site of background for atmospheric pollution in Milan. Samplings were performed on Teflon filters by a low volume gravimetric sampler (EU system, FAI Instruments, Rome, Italy). Particles were extracted from filters as previously described (Gualtieri et al., 2009); briefly, filters were detached from plastic holders and, after being immersed in sterile

water, underwent four cycles of 20 min each in an ultrasound bath (SONICA, Soltec). Particle suspensions were dried into a desiccator, weighed and stored at -20°C, and the resulting pellets were resuspended in sterile water (2µg/µl) just before use for biological analysis.

Organic extract of PM2.5 was obtained by resuspending particles in acetonitrile (CH3CN, Sigma-Aldrich) instead of water. After centrifugation (15 min, 12000 rpm, 4°C), supernatant and pellet were separated and dried into a desiccator. The organic fraction, obtained by the supernatant, was resuspended in DMSO (10µg/µl of original pellet weight), washed particles were resuspended in sterile water (2µg/µl of original pellet weight).

Chemical and morphological characterization of Milan particles has been previously and widely reported (Gualtieri et al., 2009; Perrone et al., 2010; Camatini et al., 2010) and is not showed here.

2.2. Cell culture and exposure

The SV40 hybrid (Ad12SV40) transformed human bronchial epithelial cell line BEAS-2B was purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were maintained in LHC-9 medium at 37°C with 5% of CO2, split every three day and the medium was changed the day after. For experiments, cells were seeded at a concentration of 80.000 cells/well in 6-well plates and treated after two days with 7.5 µg/cm^2 of winter PM2.5 or the equivalent amount of organic extract and washed particles. The biological responses were investigated at different time points after the treatment from 1 to 40 h, as reported in the results. For each experiment, untreated control cells were carried together.

Several inhibitors were used to investigate mechanisms of action of PM: to clarify the role of ROS, cells were pre-incubated for 1 h with N-acetyl cysteine (NAC, 10mM) and thiourea (Thio, 100µM) before exposure to

particles. Cells were exposed to alpha-naphtoflavone (α-NF; 10μM) for 1 h before particles exposure to investigate the role of AhR and Cyp enzymes. Black carbon (BC, Sigma-Aldrich, Italy) was used at the same concentration of PM (7.5 μg/cm^2) as reference carbonaceous material.

2.3. Flow cytometry

2.3.1. Cell cycle analysis

The progression through cell cycle of PM-treated cells was investigated at different time points, from 1 up to 40 h, while organic extract and washed particles effects were analysed at 3, 10 and 24 h. At 3 h, inhibition tests with α-NF and NAC were performed. For each time point, the cell cycle of untreated control cells was analysed. After the exposure, the cells have been harvested, fixed in 70% ethanol at -20°C and stored until analysis. After discharge of ethanol, cells were resuspended in PBS and RNAse DNAse-free (Sigma-Aldrich, Italy) was added for 30 min at 37°C. The fluorescent dye propidium iodide (PI) was used to stain DNA of cells and fluorescence was measured by flow cytometer using 575 nm band pass filter.

2.3.2. Cyclin B1 expression

Expression level of Cyclin B1 was assessed by flow cytometry. For measurements the cells were harvested, fixed with 1% paraformaldehyde on ice for 15 min, resuspended in cold methanol 90% and stored overnight at -80°C. After discharge of ethanol, cells were washed once in PBS 0.5% BSA and incubated with the proper primary antibody (Cyclin B1, 1:100 dilution, Cell Signaling) in PBS 0.5% BSA, 0.2% Triton X-100 overnight at 4°C. Alexafluor-488 secondary antibody (1:500 dilution, Invitrogen) was incubated for 1 h at room temperature. Finally, cells were washed once in PBS 0.5% BSA, resuspended in 500 μl PBS and analyzed on flow cytometer. Fluorescence of 10.000 events was detected using 525 nm band pass filter.

2.3.3. ROS formation

ROS formation was investigated using the fluorescent probe 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Life Science Technologies, Italy). Cells were incubated at 37°C with 5 μM of DCFH-DA in PBS for 20 min. After been washed in PBS, cells were treated with PM for 45 or 120 min, harvested and suspended in 500 μl of PBS. Analyses with organic extract or washed particles were performed at 120 min. The ROS-linked fluorescence was quantified with the cytometer EPICS XL-MCL (Beckman-Coulter) using 525 nm band pass filter. Data were analyzed using the EXPO32 ADC software (Beckman-Coulter).

2.3.4. Mitochondrial signal

The mitochondrion-selective dye MitoTracker (Invitrogen) was used to detect mitochondrial integrity. MitoTracker uptake is dependent on the mitochondrial membrane potential thus giving an indication of the mitochondrial activity.

BEAS-2B cells exposed for 24 h to 7.5 μg/cm^2 of winter PM2.5 and BC were harvested, stained with MitoTracker (30 min, 50 nM) and fluorescence of 10.000 events was detected using 575 nm band pass filter on the flow cytometer.

2.4. Fluorescence microscopy

2.4.1. Immunocytochemistry

Cells were stained for β-tubulin and γ-tubulin to analyse mitotic microtubules (MTs) and centrosomes respectively. Cells for immunocytochemical detection of proteins were prepared following the common fluorescence microscopy techniques. Briefly, cells grown on cover slips were treated with PM as described above, washed in PBS and fixed with 1% paraformaldehyde for 15 min on ice. Permeabilization and blocking were performed in PBS 0.5% BSA, 0.2% Triton X-100 for 15 min at room

temperature. Cells were then immunocytochemically labelled with primary antibodies in PBS 0.5% BSA, 0.2% Triton X-100 overnight at 4°C (β-tubulin 1:200 dilution, Cell Signaling; γ-tubulin 1:1000). Proper Alexafluor secondary antibodies (1:500 dilution, Invitrogen) were incubated for 1 h at room temperature and the DNA of cells was counterstained with DAPI. Slides were observed under a fluorescence microscope, digital images were taken and cells were counted to further describe mitotic process.

The percentage of mitotic and apoptotic cells was assessed by fluorescence microscopy in samples exposed to PM for 3, 10 and 24 h. According to nuclei morphology, 500 cells per samples were scored as interphasic (uncondensed chromatin), mitotic (condensed chromosomes) or apoptotic (typical highly condensed and fragmented nuclei) cells.

Mitotic cells (200 cells per sample) were analysed to assess the mitotic phase: according to arrangement of chromosomes and of mitotic spindle, cells were scored as pre-anaphasic (pro- and metaphase) or post-anaphasic (ana- and telophase) cells.

2.4.2. Fluorescence microscopy of living cells

ROS formation and effects on mitochondria were analysed in living cells using DCFH-DA and MitoTracker dyes respectively. ROS and mitochondria co-localization was investigated after 2 h of treatment with PM. The cells grown on cover slips were first incubated at 37°C with 5 μM of DCFH-DA in PBS for 20 min, then exposed to PM and finally stained with MitoTracker for 30min and counterstained with DAPI. Slides were observed under a fluorescence microscope (AxioLab, Zeiss), digital images were taken and co-localization signal quantified with Axioplan software. Images of mitochondria after 24 h of treatment with PM were also taken.

2.5. Single cell gel electrophoresis (SCGE)

After 1 h exposure to various inhibitors and 3 h exposure to PM, media was removed and cells were trypsinisated and re-suspended at 1 million of cells/ml in PBS. Samples were analysed for DNA damage using the comet assay. Cells dissolved in 0.68 % LMP agarose were moulded onto GelBond films attached to plastic frames to facilitate subsequent treatment steps. After lysis, films were washed and DNA strand breaks and alkali-labile sites were detected. After electrophoresis (0.8 V/cm, 30 min, pH 13.2) and neutralisation, films were fixed in ethanol and dried. Rehydrated samples were stained with SybrGold (0.08 µl/ml in TE-buffer pH 7.4, 20 minutes) and scored with Perceptives Comet IV. The level of DNA damage was expressed as tail intensity, i.e. percent fluorescence in the comet tail, relative to the total fluorescence of the comet. The SCGE assay system used was calibrated with X-rays at intervals; an increase of 5-7 percent compares to 1000 lesions per cell, induced by 1Gy of X-rays.

2.6. Statistical analyses

Statistical differences between samples were tested with one-way ANOVA and post hoc comparisons performed with Dunnett's method, by using SigmaStat 3.1 software. Statistical differences were considered to be significant at the 95% level ($p < 0.05$).

3. Results

3.1. Cell cycle alterations in cells exposed to winter PM2.5

BEAS-2B cells exposed for 24 h at 7.5 µg/cm^2 of PM showed a slight decrease of cell proliferation, but no significant cell death was seen (Fig. 1A and data not shown). We examined the cell cycle by flow cytometry at

different time points to see if the reduced proliferation were due to an accumulation of cells at a specific phase of the cell cycle.

As can be seen in Fig. 1A, PM induced a marked and significant increase of G2/M cells from 3 h to 24 h of exposure; after 3 h of treatment with particles, G2/M cells were 33.5% in PM exposed samples versus 24.7% in controls. The G2/M level decreased to control values after 40 h of PM exposure. At this time point a significant increase of subG1 cells (Fig. 1A; 13.2% in PM exposed samples versus 6.5% in controls), representing apoptotic and apoptotic/necrotic cells was seen.

In order to further describe the G2/M increase measured by flow cytometry, we investigated the amount of mitotic cells at 3, 10 and 24 h by fluorescence microscopy; cells stained for DNA and β-tubulin were scored accordingly to nuclei and spindle morphology as interphasic, mitotic and apoptotic. At 3 h the amount of mitotic cells was similar in PM-treated and control samples (Fig. 1B), thus the G2/M increase at this time point is likely due to cells delayed at G2/M transition check point. However, following 10 h of PM exposure a dramatic increase of mitotic cells was observed when compared to controls (21.3% versus 3.3%); thus, the G2/M delay observed at 3h was followed by a delay/arrest in mitosis. Interestingly, after 24 h the level of mitotic cells in PM exposed samples was back to control levels without any marked change in the relative amount of necrotic and/or apoptotic cells (Fig. 1A and B).

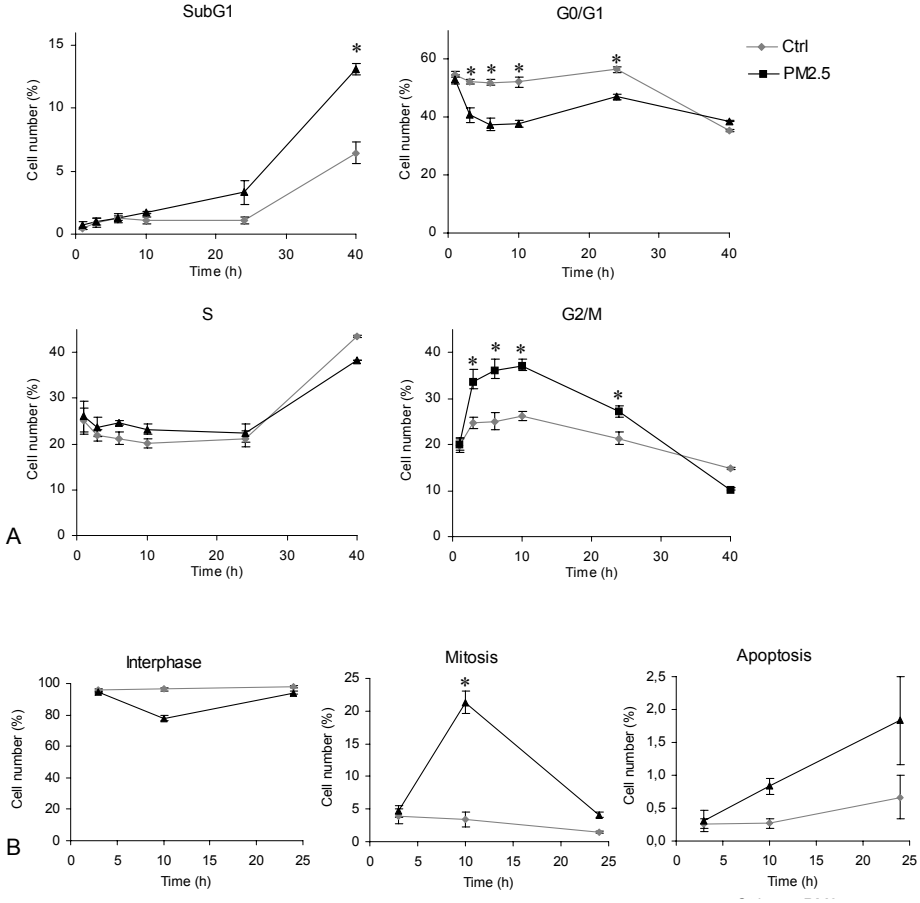


Fig. 1: Cell cycle analysis. (A) Cell cycle of BEAS-2B cells exposed to 7.5 µg/cm^2 of winter PM2.5 for 1, 3, 6, 10, 24 and 40 h. The results are representative of independent experiments (n=4). (B) Effects on mitosis valued by fluorescence microscopy: BEAS-2B cells, exposed for 3, 10 and 24 h to 7.5 µg/cm^2 of winter PM2.5, were stained for DNA and β-tubulin and scored as interphasic, mitotic or apoptotic cells. The results are representative of independent experiments (n=3), in each experiment 500 cells were scored. * Statistically significant difference from untreated cells (control), $P < 0.05$.

3.2. Characterization of the mitotic process

The mitotic process was further characterized by fluorescence microscopy: the scoring of the different phases of mitosis evidenced that, in samples exposed to PM2.5 for 10 h, post-anaphase (anaphase and telophase) occurred only in 4.3 % of the mitotic cells, compared to 31% in controls (Fig. 2). Thus, the mitotic cells in PM exposed samples seemed to be arrested at the M/A transition point.

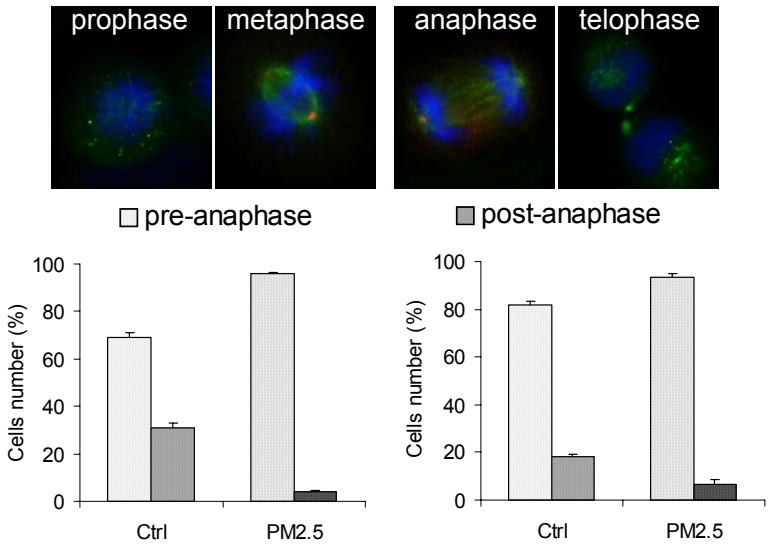


Fig. 2: Mitotic process description by fluorescence microscopy. BEAS-2B cells exposed for 10 h (left) and 24 h (right) to 7.5 μg/cm^2 of winter PM2.5, stained for DNA (blue) and β-tubulin (green) and scored as pre-anaphasic and post-anaphasic cells. Results representative of independent experiments (n=3).

Furthermore, aberrations of the mitotic spindle, represented by tripolar (Fig. 3A), multipolar (Fig. 3C) and incomplete (Fig. 3B) spindles, was also observed. Tripolar spindles accounted for 8% of mitotic cells in PM exposed

samples compared to 2% in controls and these cells seemed to be able complete the mitotic division, as anaphasic and telophasic tripolar cells were observed (Fig. 3A). Incomplete spindles were represented by bipolar spindles with groups of lagging chromosomes (Fig. 3B). In PM treated samples this configuration occurred in approximately 10% of mitotic cells while only in 1% of controls. Cells stained for γ-tubulin evidenced the presence of centrosomes amplification associated to multipolar spindles (Fig. 3C). Cells with more than 3 centrosomes represented 6.7% of mitotic cells in PM exposed samples compared to 2.7% in controls. Cells with incomplete and multipolar spindles were never found in post-anaphase.

Since the number of mitotic cells in PM treated samples was reduced to control values after 24 h (Fig. 1B) while flow cytometry still indicated enhanced G2/M levels (Fig. 1A), we analysed cell morphology also at 24 h. Fluorescence microscopy analysis revealed the presence of cells with large abnormal nuclei in samples exposed to PM for 24 h; micronuclei (MN) were detected in 18.8% of PM-treated cells compared to 3.2% of control cells and some double-nucleated cells were also observed (Fig. 4A).

As cyclin B1, in association with the cyclin-dependent kinase 1 (Cdk1), drives the progression of cells through mitosis, we next examined the level of this protein. Analysis of cyclin B1 by flow cytometry showed that this protein had significantly higher levels in cells exposed to PM2.5 for 10 and 24 h (Fig. 4B).

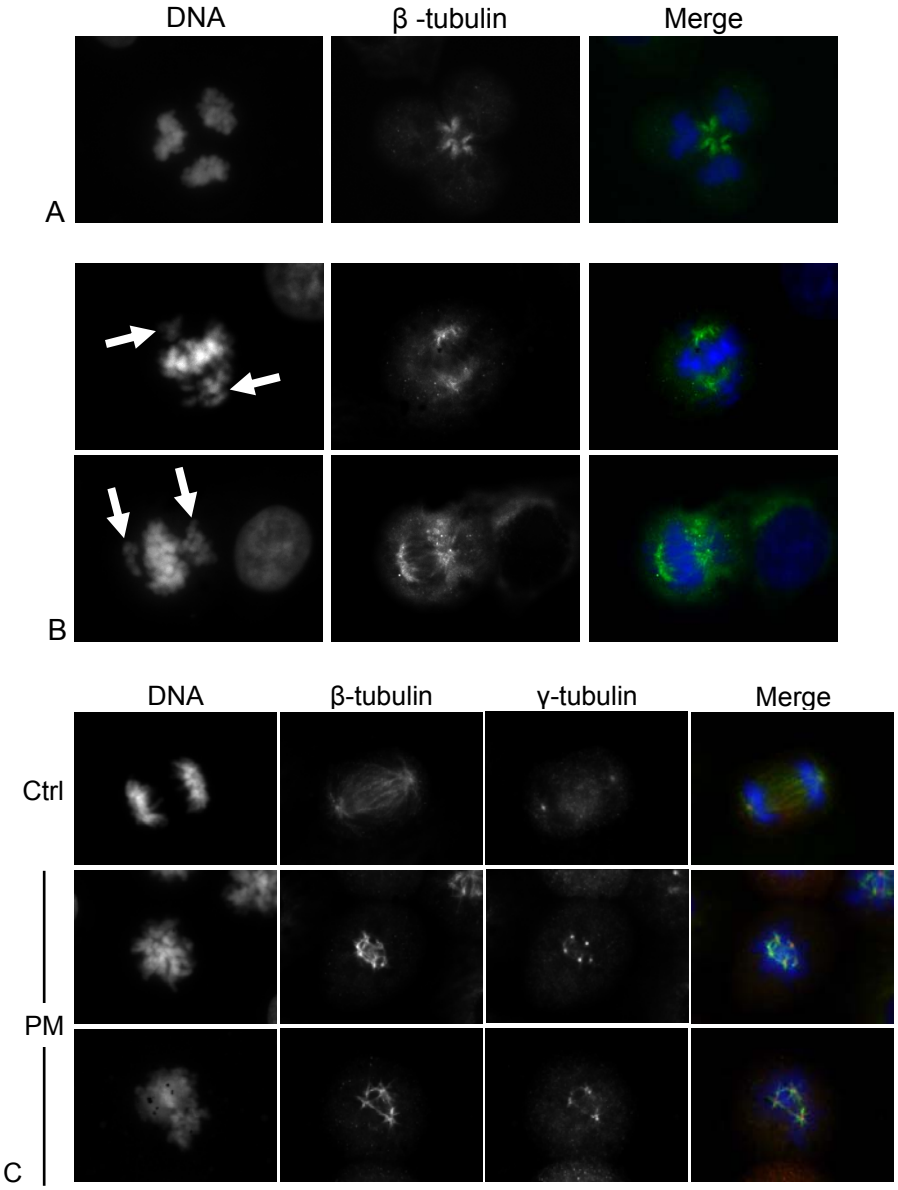


Fig. 3: Mitotic spindle analysis in cells exposed for 10 h to 7.5 μg/cm^2 of winter PM2.5: β-tubulin (green) and DNA (blue) staining showing (A) tripolar mitotic cell (telophase) and (B) bipolar incomplete spindle with groups of lagging chromosomes (arrows); (C) γ-tubulin staining (red) showing centrosomes amplification.

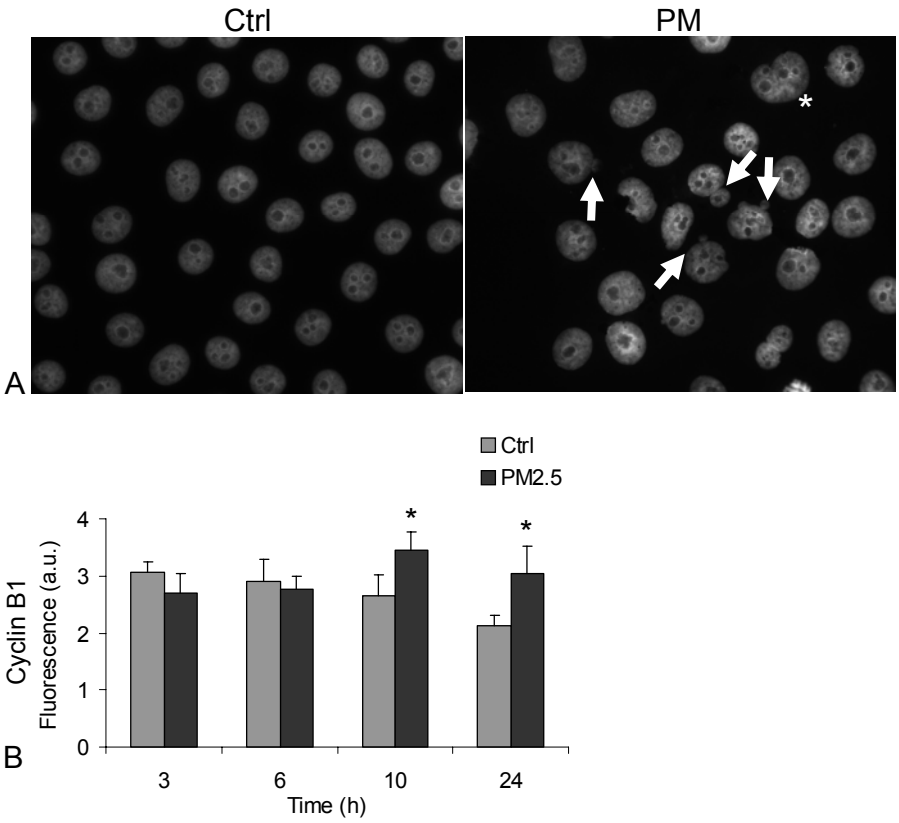


Fig. 4: Mitotic process analysis. (A) Nuclear morphology of BEAS-2B cells exposed for 24 h to 7.5 μg/cm^2 of winter PM2.5: micronucleated (arrows) and binucleated (asterisk) cells as showed by fluorescence microscopy. (B) Cyclin B1 expression measured by flow cytometry in BEAS-2B cells exposed for 3, 6, 10 and 24 h to 7.5 μg/cm^2 of winter PM2.5. The results are reported as fluorescence arbitrary units (mean ± SEM of independent experiments, n=3). * Statistically significant difference from untreated cells (control), $P < 0.05$.

3.3. Components responsible for PM-induced G2/M delay

We next tried to elucidate which components of PM were the most important for eliciting the effects observed. Organic compounds were separated from particles, and washed PM and the organic fraction tested for cell cycle

alterations. The G2/M increase induced after exposing BEAS-2B cells to organic fraction for 3 and 10 h was even higher than that observed with whole PM, while the washed particles were ineffective (Fig. 5). However, while an increased G2/M still could be observed after 24 h exposure to whole PM, rather an increase of cells in the G1 phase could be observed with organic fraction (Fig. 5).

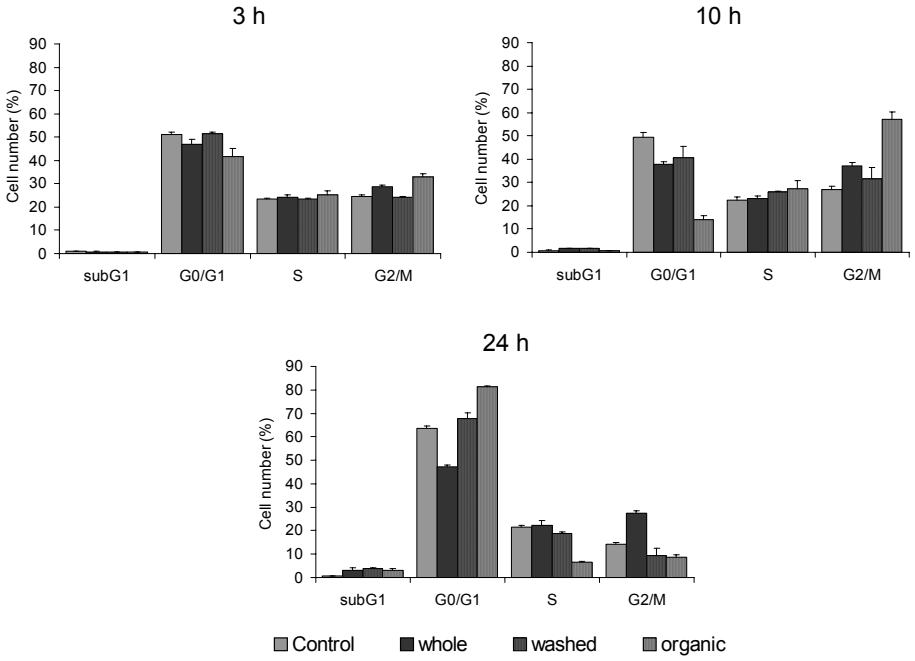


Fig. 5: Cell cycle analysis of BEAS-2B cells exposed for 3, 10 and 24 h to 7.5 μg/cm^2 of whole PM and the equivalent amount of organic fraction and washed particles. The results are representative of independent experiments (n=3).

3.4. PM-induced cellular effects relevant for G2/M delay

Then we examined PM-induced ROS formation in BEAS-2B cells in order to investigate a possible role in G2/M arrest. Noteworthy, the organic fraction induced much higher levels of ROS with the respect of whole PM,

registering a value of 22.5 a.u. versus 9.2 a.u.. Washed particles were ineffective (Fig. 6).

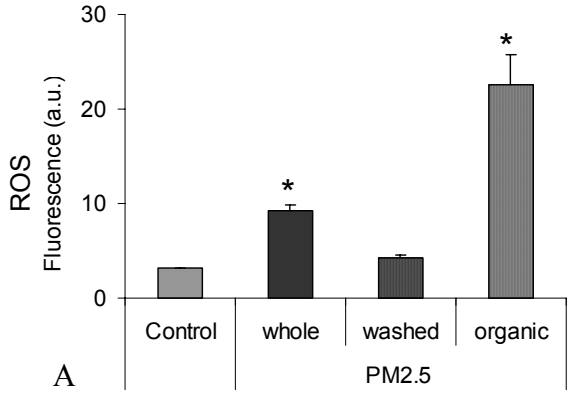
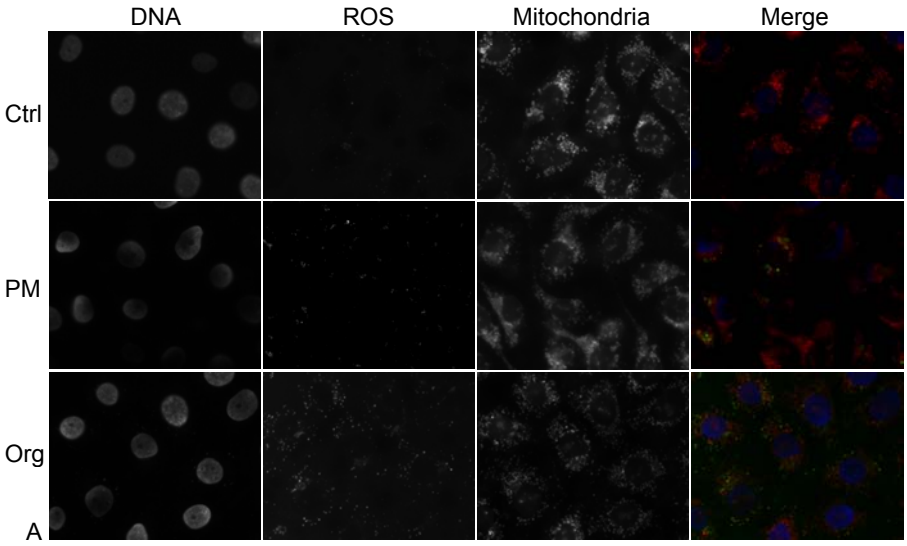


Fig. 6: ROS formation in BEAS-2B cells and effects on mitochondria. (A) Cells were exposed for 2 h to 7.5 μg/cm^2 of winter PM2.5 and to the equivalent amount of organic fraction and of washed particles. The results are reported as fluorescence arbitrary units (mean ± SEM of independent experiments, n=3). * Statistically significant difference from untreated cells (control), P< 0.05.

Since mitochondria are known to be both source and target of PM-induced ROS (Soberanes et al., 2009), we investigated the role of these organelles in ROS formation here reported. The co-localization of ROS and mitochondria was assessed by fluorescence microscopy in cells exposed for 2 h to PM2.5 and its organic fraction, by staining with DCFH-DA and MitoTracker respectively. The results showed ROS as green dots spread in the cytoplasm and partially overlapping with red fluorescence of mitochondria (Fig. 7A). The measurement of the co-localization signal, performed by Axiovision software, revealed that approximately 40-50% of ROS localized at mitochondrial level. Thus, besides the mitochondrial pathway, Milan PM also induced ROS formation directly or through other mechanisms.

The presence of mitochondrial damage was observed by fluorescence microscopy in cells treated for 24 h with PM and a statistically significant reduction of mitochondrial fluorescence signal was measured by flow cytometry (Fig. 7B). Carbonaceous particles (BC) alone produced a slight non significant decrease of mitochondrial signal, suggesting that the chemical composition of PM is responsible of this effect.



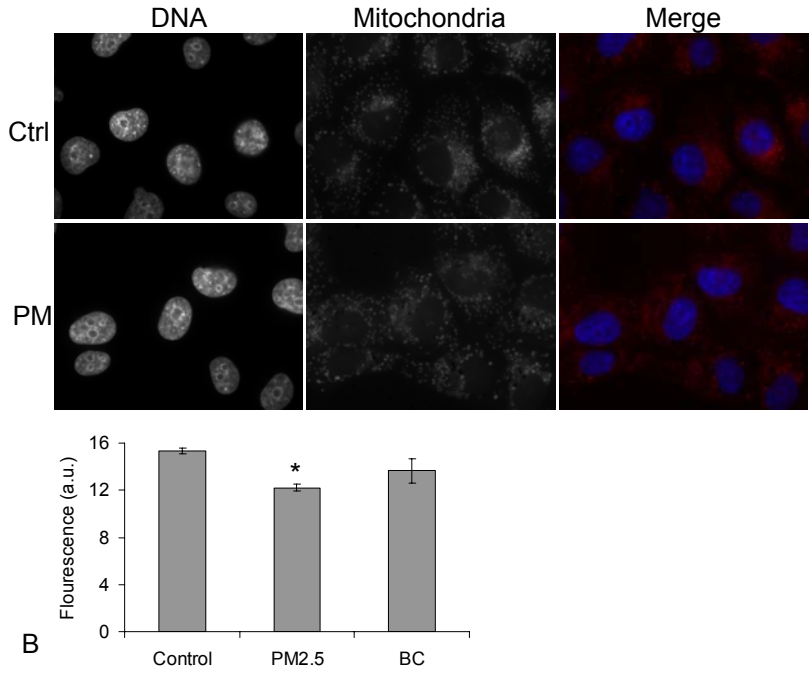


Fig. 7: (A) ROS and mitochondria co-localization as assessed by fluorescence microscopy. BEAS-2B cells, exposed for 2 h to 7.5 μg/cm^2 of winter PM2.5 and to its organic fraction, were stained for DNA, ROS and mitochondria. Co-localization signal of ROS and mitochondria was measured by Axioplan software for fluorescence microscopy as reported in materials and methods. (B) Mitochondrial damage in BEAS-2B cells exposed for 24 h to 7.5 μg/cm^2 of winter PM2.5 as assessed by fluorescence microscopy and flow cytometry. Flow cytometry results are reported as fluorescence arbitrary units (mean ± SEM of independent experiments, n=3). Black carbon (BC) was used as reference control for carbonaceous particles effects. * Statistically significant difference from untreated cells (control), $P< 0.05$.

Chemical-induced cell cycle arrest is known often to be related to DNA damage. Thus, we examined the genotoxic potential of winter PM2.5, measured as DNA strand breaks under alkaline conditions (SCGE-assay).

The possible role of reactive electrophilc or radical metabolites, including ROS, were explored by the addition of the nucleophilc and antioxidant molecules N-acetylcysteine (NAC) and thiourea (Thio); the possible role of cyp450-dependent metabolism was studied by the addition of the CYP-inhibitor α-naphthoflavone (α-NF).

The results presented in Fig. 8 showed that PM induced DNA damage already 3 h after exposure, as seen by increased tail intensity. This damage seemed to be related both to CYP-induced reactive metabolites and/or ROS formation as α-NF as well as the nucleophilic groups/antioxidants almost completely reduced this response. Preliminary data using the addition of fpg, converting 8-oxo-G to DNA-alkalilable sites (data not shown), suggested that the particle also increased oxidative DNA damage in accordance with previous observations after 24 h exposure (Gualtieri et al., 2011a).

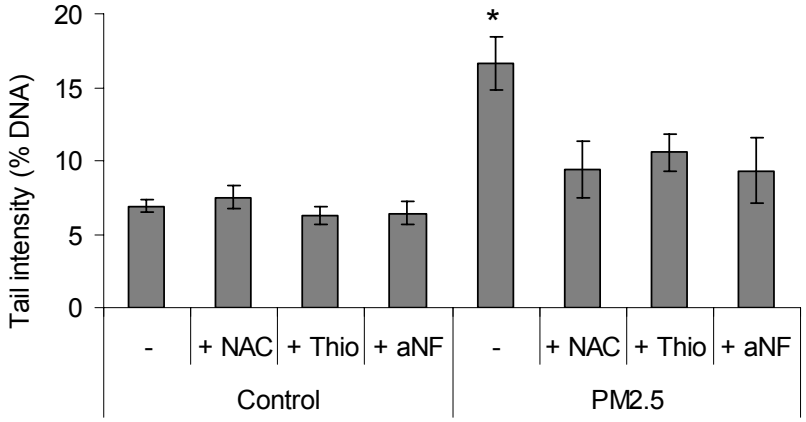


Fig. 8: DNA damage in BEAS-2B cells exposed for 3 h to 7.5 μg/cm^2 of winter PM2.5 by SCGE assay. NAC, Thio and α-NF were used to determine the role of ROS and CYP enzymes respectively. DNA-damage was expressed as tail intensity (% DNA in tail). * Statistically significant difference from untreated cells (control), $P < 0.05$.

Moreover α-NF and NAC completely abolished the PM and organic fraction-induced G2/M accumulation after 3 h of exposure (Fig. 9). This effect of NAC and α-NF, further supports an important role for a cyp450 linked activation of components occurring in the organic fraction.

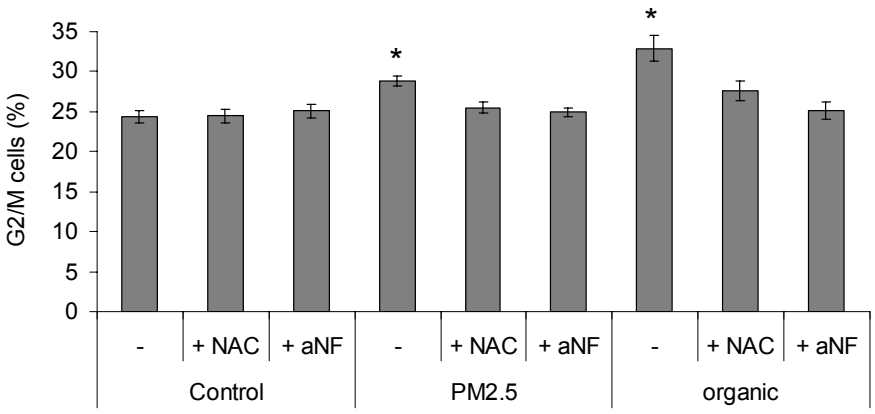


Fig. 9: G2/M cells percentage assessed by cell cycle analysis of BEAS-2B cells exposed for 3 h to 7.5 μg/cm^2 of whole PM and organic fraction. NAC and α-NF were used to determine the role of ROS and CYP enzymes respectively. The results are representative of independent experiments (n=3).

4. Discussion

We have previously reported that Milan winter PM2.5 may result in mitotic catastrophe. Here we show that lower doses of winter PM2.5 induce cell cycle delays, first in G2 phase, then at the M/A transition point, finally resulting in tetraploid G1 cells and cells with increased number of micronuclei. The effects seemed to be related to chemicals in the organic fraction of PM, that were metabolic activated via cyp-450 enzymes to

reactive electrophilic/radical metabolites damaging mitochondria, chromosomal spindle as well as the DNA itself.

Rather low concentrations of winter PM2.5 (7.5μg/cm^2) rapidly (from 3 h) induced an increase of cells arrested in G2/M that was observed up to 24 h. This accumulation probably reflects a delay in cell cycle, as the cell number after 24 h was reduced (data not shown). No mitotic-apoptosis as previously reported after 24 h exposure to a higher dose of Milan winter PM2.5 (25 μg/cm^2; Gualtieri et al., 2011a) could be seen at this dose. Reduced cell proliferation following PM-exposure has been previously reported (Kochbac et al., 2008; Poma et al., 2006; Möller et al., 2002), and in some cases it has been found to be related to arrest in specific points of the cell cycle (Kochbac et al., 2008; Gualtieri et al., 2011a). The point of arrest may vary and *in vitro* studies have reported both G1, S and G2/M arrest in cells treated with PM. These delays have mainly been associated to PM-induced DNA damage and following DNA damage response (Deng et al., 2007; Zhang et al., 2007; Jalava et al., 2007; Kochbac et al., 2008; Gualtieri et al., 2011a).

With regard to the present study, it is of pivotal importance to clarify if the observed G2/M cells are in G2 or M phase to explore possible mechanism that leads to the arrest. In fact cell cycle progression can be blocked or delayed not only in response to genotoxic stresses, but also to structural dysfunctions of various proteins (Rieder, 2011). As a part of this characterization we found that the cells were arrested/delayed at different steps of the cell cycle; the combined use of flow cytometry and fluorescence microscopy revealed an early increase of G2 cells after 3 h of exposure followed by mitotic arrest/delay at 10 h. Finally also the cytokinesis was affected since non-mitotic diploid cells (4N) were observed at 24 h. Significantly increased levels of cell death were observed after 40 h of

exposure, as a likely result of the mitotic catastrophe. Thus, these observations suggest that several types of initial damage may be involved, including genotoxic stress as well as structural dysfunctions linked to microtubule and mitotic spindle organization.

G2 arrest/delay in cells is due to the activation of G2/M transition checkpoint, a rapid-response system that does not require transcription and translation and thus can be quickly switched on (Rieder, 2011). It is known that this checkpoint can be activated by DNA damage, through the ATM/ATR pathway, and by other stresses such as MTs disassembly, topoisomerase II inhibition and histone deacetylation, through the pathway of p38 kinase. Both these pathways depress the activity of cyclin A- and B-dependent kinases thus preventing the ingress into mitosis (Fukasawa, 2008). Here we found that PM induced G2 arrest was accompanied with DNA damage. Furthermore, αNF and NAC reduced DNA damage as well as the accumulation of cells in G2, confirming the role of DNA damage in the induction of cell cycle arrest and suggesting that reactive metabolites are linked to this process. We previously reported that Milan winter PM2.5 causes the activation of the DNA damage response involving the ATM/ATR pathway and p53 in BEAS-2B cells (Gualtieri et al., 2011a) and enhanced levels of these proteins have been observed in A549 cells exposed to PM (Sánchez-Pérez et al., 2009) and to cigarette smoke at very early time points (20 min; Tanaka et al., 2007). These data confirm the involvement of PM-induced DNA damage and a DNA damage response in eliciting the effects on cell cycle but they do not exclude that other kind of stresses pathways, including stress activated p38 (Rieder, 2011), may be involved. Actually the complex composition of PM may determine the contemporary activation of various pathways.

A rapid G2 block is primarily induced in a transient way and requires p53/p21 transcription to ultimately produce a sustained block (Rieder, 2011). However, we observed a transient delay of G2 that was no more present after 10 h of treatment, when cells arrested/delayed in mitosis were found. This outcome suggests that the cells delayed in G2 at 3 h escaped from this block without properly repair the damage, thus undergoing mitosis in presence of severe DNA and/or mitosis machinery lesions (Wang et al., 2009). At 10 h of exposure, almost all mitotic cells were delayed in pro/metaphase and some of them showed various aberrations of the mitotic apparatus represented by tripolar, multipolar and incomplete spindles, suggesting that PM may act as spindle poison, directly perturbing microtubules (MTs) dynamics. These kinds of spindle aberrations have been also reported by Glowala et al. (2002) in Chinese hamster fibroblasts exposed to PM10; the authors did not investigated the factors responsible for induction of spindle defects but suggest a role of mutagens and/or compounds in PM that may interfere with microtubule assembly/disassembly or affect components of centrosomes (Glowala et al., 2002).

The presence of aberrant spindles and M/A arrest indicate the involvement of the SAC. In fact centrosomes amplification, and the consequent increased number of spindle poles, has been reported to delay anaphase onset through SAC activation, allowing other mechanisms to cluster the centrosomes and enabling a bipolar spindle to form (Acilan and Saunders, 2008). SAC can be also activated by the presence of incomplete bipolar spindles with lagging chromosomes, as the ones we reported; pole-associated chromosomes are a regular transient feature of astral spindle assembly, when monotelic attachment of the chromosomes results in motion toward the centrosomes, but this feature should be rapidly corrected by an Aurora-B kinase-based mechanism (Rieder, 2011). The presence of a high percentage of cells with pole-associated chromosomes (10% in PM-treated samples) suggests delays

in the rearrangement of the attachment, which might cause the activation of SAC.

Non-mitotic 4N cells were found in samples exposed to PM for 24 h and after 40 h of exposure we observed an increased level of subG1 cells, representing cell death. Thus the cells arrested in mitosis at 10 h likely were not able to correct the errors and underwent mitotic slippage and subsequent mitotic catastrophe. The presence of micronuclei and double nuclei observed in 24 h exposed cells sustain this hypothesis since it is known that cells transiently arrested by SAC at the M/A transition point can exit mitosis without proper segregation of DNA and cytokinesis, if the damages that caused the checkpoint activation are not corrected in a certain period of time. This process, named mitotic slippage, gives rise to 4N G1 cells, which besides having large nuclei usually present multiple micronuclei (Castedo et al., 2004). In agreement with observations in literature (Fukasawa, 2008), we found that cells with amplified centrosomes that form tripolar mitotic spindles most likely underwent cytokinesis, as tripolar cells in anaphase and telophase were observed. On the contrary, cells with more than three poles were never found in these late phases of cycle suggesting that they fail cytokinesis resulting in binucleated or micronucleated cells.

Cells exposed for 24 h to PM also seemed to have high levels of cyclin B. This is well in agreement with the fact that activation of SAC inhibits the APC-dependent degradation of cyclin B and thus maintaining high levels of this protein. Moreover it has been demonstrated that cyclin B degradation not only is required for the transition to anaphase, but also to the onset of cytokinesis in *Drosophila* (Echard and O'Farrell, 2003). Interestingly, Burns et al. (2003) found high levels of cyclin B1 in 4N cells treated with nocodazole and paclitaxel. On the other hand, Brito and Rieder (2006)

reported that cyclin B degradation is required for mitotic slippage to occur. The role of cyclin B in mitotic slippage onset is thus still controversial.

The presence of spindle aberrations, mitotic slippage and subsequent mitotic catastrophe here reported suggests that PM may acts as a spindle poison, as well as DNA damaging agent, to induce cell cycle alterations and finally cell death. Urban dust and diesel exhaust particles (DEP) have been previously reported to cause cytoskeletal dysfunctions, including effects on phagocytosis, phagosome transport mechanisms, cytoskeletal integrity. These effects were observed in alveolar macrophages and seemed to be dependent on the organic fraction (Möller et al., 2002). We previously reported high levels of cell death to occur by mitotic catastrophe after 20 to 24 h of exposure to 25 μg/cm^2 of winter PM2.5. Here we exposed the cells to a lower dose of PM (7.5 μg/cm^2) and observed significant levels of cell death after 40 h. These results are in line with the typical behaviour of spindle poisons; in fact it has been showed that cells exposed to low poison concentrations might escape mitosis and form 4N G1 cells that remain viable for long periods while, when exposed to high drug concentrations they die in, or shortly after, mitosis (Rieder et al., 2011). All these are protective mechanisms that impede incorrect cell divisions possibly leading to aneuploidy, a dangerous condition since it is a common step of many cancers (Wang et al., 2009). However, in PM-treated samples some tripolar cells were found to be able to satisfy the SAC and complete the mitotic division, as anaphasic/telophasic tripolar cells were observed, and such events might actually give rise to aneuploid cells. Thus the possibility of such events to occur in PM treated cells should be further investigated.

The results here reported show that organic fraction is the sole responsible of the effects of PM, while washed particles were ineffective regarding the

outcomes investigated. Organic fraction induced a rather similar G2/M accumulation in BEAS-2B cells as seen for PM. ROS level was ever more enhanced by organic fraction than seen by whole PM. Metabolic biotrasformation of organic compounds to reactive electrophilic metabolites and/or ROS formation appeared to drive the DNA damage induction and G2 arrest, as demonstrated by the use of antioxidants and αNF, an AhR/CYP enzymes inhibitor. Several studies have investigated the role of organic compounds coated onto PM in inducing toxicity (Chackra et al., 2007; Nel et al., 2001; Billet et al., 2007). In most of these reports high PAHs content has been associated and linked to the high genotoxicity, oxidative stress and cytoskeletal dysfunctions (Sevastyanova et al., 2007; Binkova et al., 2003; Möller et al., 2002). Effects on the cell cycle have been reported for PAHs-rich PM0.2 resulting from combustion of solid fuels that has been shown to induce G2/M arrest in macrophages (Jalava et al., 2007), while organic extracts from PM2.5 and PM10 arrested different human cell lines in G0/G1 (Deng et al., 2007; Dong and Yang, 2005). Several PAHs are known to alter cell cycle in various ways; dibenzo[a,l]pyrene induces G2/M arrest in human mammary carcinoma MCF-7 cells (Baird et al., 1999), while dibenzo[a,l]pyrene and benzo[a]pyrene delay HEL fibroblasts in the S phase (Binkova et al., 2000). Thus, PAHs in the PM might differently affect the cells cycle much depending on the specific compounds present, and the metabolic capacity of exposed cells. The enhanced bioavailability of the organic compounds for cells after their extraction from particles might explain the higher toxic potential of PM organic fraction and the somewhat enhanced response here seen.

ROS formation is a well recognized primary effect of PM exposure promoting various secondary effects including inflammation and DNA damage (Li et al., 2008; Schwarze et al., 2006; de Kok et al., 2006).

Moreover, high levels of oxidants involve perturbation of the mitochondrial permeability and disruption of electron transfer that result in cellular apoptosis or necrosis (Li et al., 2008). Mitochondria have been indicated as the main source of ROS generation in rats alveolar type II and A549 cells exposed to a quite high dose of PM2.5 (50 μg/cm^2) (Soberanes et al., 2009). However, our data showed that only one part (40-50%) of ROS can be attributed to mitochondria after exposure to a much lower dose of PM (7.5 μg/cm^2), indicating that different mechanisms, besides mitochondrial pathway, likely concur to ROS formation. Besides mitochondria, metabolic activation of PAHs through cytochromes P450 (CYP) and AKR enzymes is known to induce ROS formation catalyzing the oxidation of PAHs to quinones, electrophilic metabolites that enter redox cycles and amplify ROS (Burczynski and Penning, 2000). Accordingly, we found that organic fraction induced higher levels of ROS compared to PM. Furthermore, the reactive electrophilic PAHs intermediates produced by the CYP and AKR enzymes are known to induce DNA damages in an AhR-dependent way (Park et al., 2009); accordingly, AhR-response has been found to be of major importance in explaining the toxicity of the organic PM fraction (Andrysik et al., 2011). We previously reported the activation of AhR/CYP pathway in A549 cells exposed to Milan winter PM2.5 (Gualtieri et al., 2011b) and increased levels of CYP1A1 in BEAS-2B cells (Gualtieri et al., 2011a). Here we demonstrated the role of AhR/CYP pathway and reactive electrophilic metabolites and/or ROS in PM-triggered DNA damage and cell cycle alteration, by the use of the CYP enzymes inhibitor αNF and antioxidants NAC/Thiourea.

In conclusion, our study shows that rather low doses of winter PM2.5 induced an early G2 arrest which was followed by an arrest in metaphase/anaphase (M/A) and by an inhibition of cytokinesis and increased

formation of diploid cells with micronuclei. These effects seemed to be caused by chemicals in the organic fraction and to be linked to DNA and/or mitotic spindle damage. DNA damage and G2/M accumulation were linked to the formation of reactive electrophilic/radical metabolites via a cyp450 depended reaction. Moreover we reported the ability of PM-induced tripolar cells to undergo cell division, thus leading to aneuploid cells which are considered a preliminary step in cancer development.

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Supplementary data I

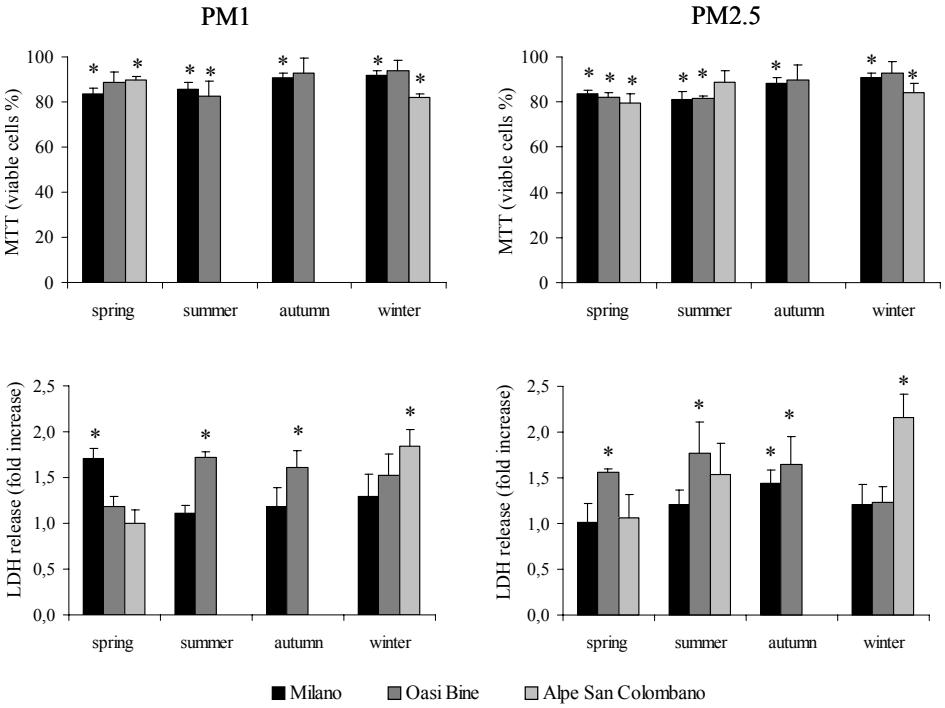

Fig. 1: A549 cell viability measured by MTT test and LDH release after 24 h exposure to 6 μg/cm^2 of PM1 and PM2.5 sampled in different seasons. Data are expressed as mean ± SEM. * Statistically significant difference from control (ANOVA; $p<0,05$).

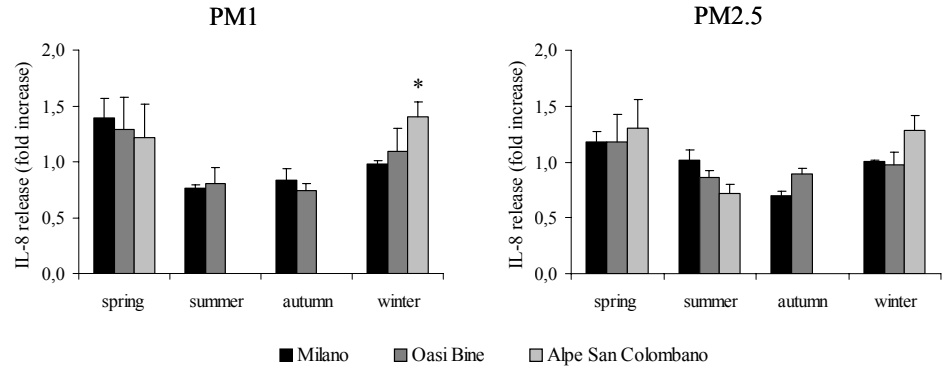

Fig. 2: IL-8 release in A549 cells exposed for 24 h to 6 µg/cm^2 of PM1 and PM2.5 sampled in different seasons. Data are expressed as mean ± SEM. * Statistically significant difference from control (ANOVA; p<0,05).

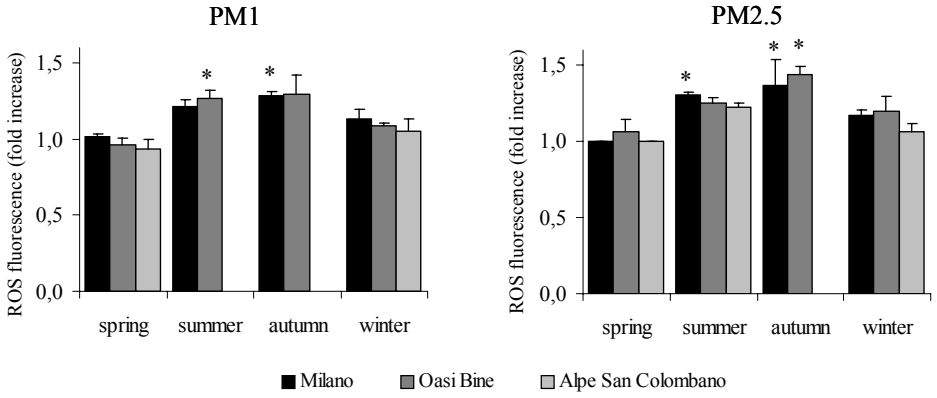

Fig. 3: ROS formation in A549 cells exposed for 2 h to 6 µg/cm^2 of PM1 and PM2.5 sampled in different seasons. Data are expressed as mean ± SEM. * Statistically significant difference from control (ANOVA; p<0,05).

Supplementary data II

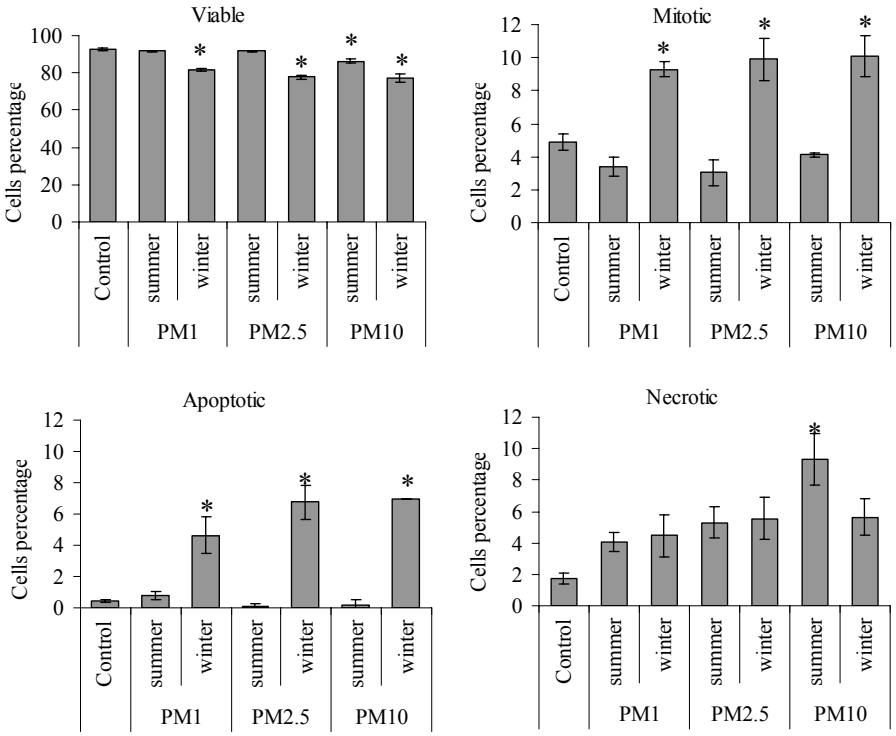

Fig. 1: Viable, mitotic, apoptotic and necrotic THP-1 cells as scored after Hoechst/PI staining after 24 h exposure to 25 μg/cm^2 of PMs. Data are expressed as mean ± SEM. * Statistically significant difference from control (ANOVA; p<0,05).

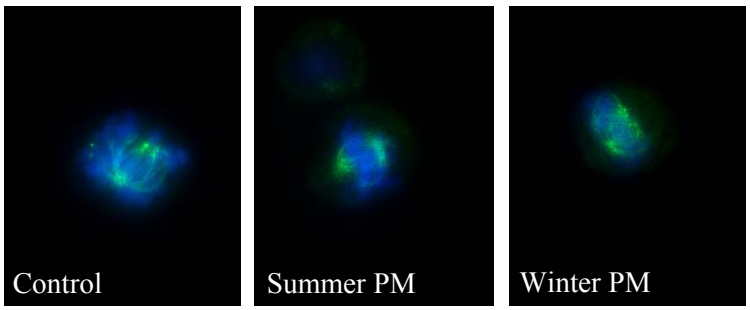

Fig. 2: THP-1 mitotic cells stained for β-tubulin (green) and DNA (blue) after 24h exposure to 25μg/cm^2 of PMs. While summer PM2.5 treated cells presented normal spindles, winter PM2.5 treated cells had abnormal spindles.

Supplementary data III

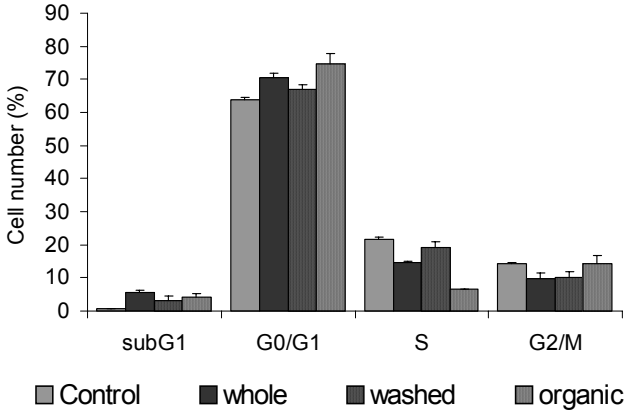

Fig. 1: Cell cycle analysis of BEAS-2B cells exposed for 24 h to 7.5 μg/cm^2 of whole PM1 and the equivalent amount of organic fraction and washed particles. An accumulation of cells in G1 phase can be observed in sampled exposed to whole PM1 and its organic fraction. The results are representative of independent experiments (n=3).

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