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Integrative transcriptomic and protein analysis of human bronchial BEAS-2B exposed to seasonal urban particulate matter

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ABSTRACT

Background: Exposure to particulate matter (PM) is associated with various health effects. Physico-chemical properties influence the toxicological impact of PM, nonetheless the mechanisms underlying PM-induced effects are not completely understood.
Objectives: Human bronchial epithelial cells were used to analyse the pathways activated after exposure to summer and winter urban PM and to identify possible markers of exposure.
Methods: BEAS-2B cells were exposed for 24 h to 10 μg/cm^2 of winter PM2.5 (wPM) and summer PM10 (sPM) sampled in Milan. A microarray technology was used to profile the cells gene expression. Genes and microRNAs were analyzed by bioinformatics technique to identify pathways involved in cellular responses. Selected genes and pathways were validated at protein level (western blot, membrane protein arrays and ELISA).
Results: The molecular networks activated by the two PM evidenced a correlation among oxidative stress, inflammation and DNA damage responses. sPM induced the release of pro-inflammatory mediators, although miR-146a and genes related to inflammation resulted up-regulated by both PM. Moreover both PM affected a set of genes, proteins and miRNAs related to antioxidant responses, cancer development, extracellular matrix remodeling and cytoskeleton organization, while miR-29c, implicated in epigenetic modification, resulted up-regulated only by wPM. sPM effects may be related to biological and inorganic components, while wPM apparently related to the high content of organic compounds.
Conclusions: These results may be helpful for the individuation of biomarkers for PM exposure, linked to the specific PM physico-chemical properties.

1. Introduction

Particulate matter (PM) is nowadays considered one of the major environmental risk to health in the world, responsible for 3.7 million annual premature deaths (WHO, 2014) for induction of pulmonary and cardiovascular diseases, and lung cancer (Loomis et al., 2013; Kim et al., 2015). Despite the international research effort, several aspects concerning the biological mechanisms responsible for PM-induced health effects remain unclear. Recent studies have underlined the role of PM physico-chemical characteristics in promoting its specific biological outcomes. Moreover several reports (Cassee et al., 2013; Kim et al., 2015; Rohr and Wyzga, 2012) suggest that the presence of certain PM components may be more significant than PM concentration itself in inducing health effects. Nevertheless researchers are far from having a detailed mechanistic explanation of the causal relation between PM and health effects suggested by epidemiological

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evidences.

Toxicological studies on *in vitro* cell systems are crucial to investigate the cellular mechanisms and provide support to clinical and epidemiological observation (Huang, 2013). Oxidative stress, inflammation and DNA damage are the main PM-induced cell outcomes (Nemmar et al., 2013). All these processes have an important role in eliciting asthma, allergy, chronic obstructive pulmonary disease (COPD), cardiovascular diseases and cancer, however the molecular pathways involved can be several and related to different biological mechanisms.

Accumulating evidences (Nel, 2005; Fahmy et al., 2010) demonstrate that fine PM, namely particles with mean aerodynamic diameter lower than 2.5 microns ($PM_{2.5}$), mainly composed of combustion-derived particles with a high redox capacity, polycyclic aromatic hydrocarbons (PAHs) and metals, is responsible for the different health effects. Epidemiological data report that the exposure to vehicular emissions is associated to cardiovascular mortality and morbidity, asthma exacerbation and other respiratory diseases (Rohr and Wyzga, 2012). These results are confirmed by toxicological studies on diesel emissions, diesel exhaust particles (DEP), and particulate samples collected near highways in high trafficked cities, demonstrating that fine PM induces biological responses related to health diseases (Ghio et al., 2012; Grahame and Schlesinger, 2010). The importance of the organic fraction adsorbed on fine PM in determining cell damages has been extensively analysed and significant activation of signalling pathways and gene expression reported (Andrysík et al., 2011; Líbalová et al., 2014).

Besides, a positive associations between coarse PM (PM10−2.5) and respiratory and cardiovascular diseases and mortality has been evidenced by epidemiological data (Cassee et al., 2013; Huang et al., 2011; Kelly and Fussell, 2012). Coarse PM is dominated by mechanically abraded particles, minerals, metals and biological components, which play a central role in health effects, stimulating the alveolar macrophages and the respiratory cells to release pro-inflammatory cytokines and chemokines (Nemmar et al., 2013). It has also been reported that biological compounds may act together with other PM components, such as diesel exhaust, to enhance IgE production and promoting allergic sensitization (Schwarze et al., 2010). Moreover the presence of transition metals with a strong redox activity, such as vanadium and nickel (residual oil fly ash), iron, manganese and molybdenum (engine abrasion), and copper or antimony (brake wear), determines an increment of PM10 oxidative potential (Cassee et al., 2013).

The complex PM's chemical composition is responsible for our lack of understanding of the causal relation between particles inhalation and human diseases; due to this complexity, the possibility to uncover the mechanisms of action require the use of high throughput approaches in support of classical toxicological analyses.

During these last years “omics” approaches have been used to investigate complex molecular mechanisms. Toxicogenomics, including in also transcriptomics, has demonstrated its utility to assess the toxicity of drugs on human cell lines and to clarify the action mechanism of new compounds (Hartung, 2009; Jennings et al., 2013). Global gene expression profiling has been increasingly used to investigate PM-associated health effects; gene modulation was found in pathways related to oxidative stress, metabolism of xenobiotics, inflammatory cytokines and Toll-like receptor signalling (Huang, 2013), supporting the toxicological evidences of PM biological effects. Other pathways related to i) cell cycle regulation, ii) apoptosis, iii) mitogen-activated protein kinase (MAPK) signaling, iv) T- and B-cell receptor signaling, v) metal binding, vi) cell aging and vii) cancer development, resulted modulated in relation to the particles chemical composition. Moreover the changes in miRNA expression have been suggested to be a novel mechanism involved in PM response (Bollati et al., 2010) and miRNAs have been proposed as novel indicators of environment exposure (Vrijens et al., 2015). Therefore the transcriptomic approach, integrating gene and miRNA analysis, can be useful to find the biological linkage between PM health effects and the promoting compounds.

The present study intends to evaluate the signaling pathways associated to winter PM2.5 (wPM) and summer PM10 (sPM), on human bronchial BEAS-2B cells by means of a global transcriptomic profiling and to investigate selected pathways at protein level. MiRNAs have also been analyzed to identify their role in PM-induced effects and to evaluate their potential as markers of exposure.

2. Material and methods

2.1. PM collection and preparation

Summer PM10 (sPM) and winter PM2.5 (wPM) samples were collected at Torre Sarca (Milan), a representative urban site. Sampling and detaching of particles from filters were performed as previously reported (Gualtieri et al., 2012). The mean particles recovery in the present study was of 75%, as determined by the ratio of mass of particles collected after extraction over the mass of particles sampled on filters.

2.2. Cell culture and treatments

The human bronchial epithelial BEAS-2B cells (European Collection of Cell Cultures, ECACC, Salisbury, UK) were maintained in LCH-9 medium (Gibco-Invitrogen) and plated on collagen (PureColTM)-coated flasks. Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2; the medium was replaced every two days. Cells were seeded into 6-well culture plates at 80,000 cells/well and treated after 48 h with 10 μg/cm^2 of summer PM10 or winter PM2.5 for 24 h.

2.3. RNA extraction

Cells were lysed and stored in QIAzol Lysis reagent (Qiagen, Hilden, Germany) until the RNA extraction. Total RNA was extracted from cells using the miRNeasy extraction kit (Qiagen, Hilden, Germany) and eluted in RNase free-water, according to the manufacturer's recommended guidelines. RNA samples quantification was performed using ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the quality was checked with 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples with RIN (RNA Integrity Number) $\geq$ 9 and presence of low molecular weight RNA molecules (including 5S rRNA and small RNAs) were considered for the experiments. 1 μg of total RNA was used for gene and microRNA expression profiling.

2.4. Global gene expression analysis by GeneChip

Gene expression were analysed in the human bronchial epithelial cell line, BEAS-2B exposed to wPM and sPM trough microarray analysis. Unexposed cells were used as control and the experiment was performed in triplicate. Biotin-labelled target was prepared from total RNA according to GeneChip® Expression Analysis Technical Manual protocol (Affymetrix, Santa Clara, CA, USA). Samples were fragmented and 15 μg of the obtained cRNA were hybridized at 45 C° for 16 h onto GeneChip® Human U133 Plus 2, nonspecifically bound material was removed by washing. Specifically bound targets were detected using GeneChip®

Hybridization, Wash and Stain kit, and the GeneChip® Fluidics Station 450 (Affymetrix). Fluorescent images of the array were scanned with GeneChip® Scanner 3000 7G (Affymetrix) producing raw data. Raw data (files.cel) are available in the Array express database (E-MTAB-3629) were processed and normalized using Partek GS we carried out the Analysis of Variance (ANOVA) on the entire data set (9 samples, including three groups). Two lists of differentially expressed genes were generated using an FDR (Benjamini Hochberg) of <0.05 and a cut off for [2] fold change (FC).

2.5. Global microRNA expression analysis by GeneChip

MicroRNA expression were analysed in the human bronchial epithelial cell line, BEAS-2B exposed to wPM and sPM trough microarray analysis. Unexposed cells were used as control and the experiment was performed in triplicate. Total RNA samples were labelled using the FlashTag Biotin RNA labeling kit (Genisphere Inc., Hatfield, PA) following the manufacturer's instructions and then hybridized onto the arrays at 48 °C for 16 h. Affymetrix GeneChip miRNA Array 2.0 (Affymetrix, Santa Clara, CA, USA) probe sets include all the 1100 human microRNAs (has-miRNAs) in miRBase registry (http://www.mirbase.org). Fluidics Station 450 was used for washing and staining with Hybridization Wash and Stain Kit (Affymetrix). Array fluorescent images were acquired with GeneChip Scanner 3000 7G and analyzed using GeneChip Operating Software. Raw data (files.cel) were processed and normalized using Partek GS. The Analysis of Variance (ANOVA) on the entire data set (9 samples, including three groups) was carried out. Two lists of differentially expressed microRNAs (DEMs) were generated setting a P-value < 0.05 and a cut off for [2] fold change (FC). Data (CEL files) discussed in this publication have been deposited in ArrayExpress repository (http://www.ebi.ac.uk/arrayexpress/) and are available through the accession number E-MTAB-3630.

2.6. Functional enrichment analysis of differentially expressed genes

To analyze lists of genes that were differentially expressed in BEAS cells after exposure ToppFun tool (Chen et al., 2009) was performed. Functional enrichment analysis basing on several categories was obtained; biological process and pathways as functional categories was considered for the analysis. ToppFun tool determined a statistical significance using hypergeometric distribution with Bonferroni correction and a p-value threshold ≤0.05.

2.7. Bioinformatics study of differentially expressed microRNAs

General information about DEMs was collected in miRbase. miRbase databank (http://www.mirbase.org/) contains all the published sequences and annotation of known microRNAs. In order to investigate validated or predicted targets of selected microRNAs miRwalk database was used (Dweep et al., 2014) (http://mirwalk.uni-hd.de/). MiRandola database (Russo et al., 2014) (http://atlas.dmi.unict.it/mirandola/) was used to obtain information about extracellular circulating microRNA and select potential biomarkers. To obtain evidence for human miRNAs and disease associations the Human microRNA Disease Database v. 2.0 was used (HMDD) (Li et al., 2013) (http://www.cuilab.cn/hmdd).

2.8. Real time quantitative polymerase chain reaction (qPCR)

qPCR was performed using the High capacity cDNA reverse Transcription Kit. Human PrimePCR™ SYBR® Green Assays (Bio-Rad) were used to study the following genes: *UGT1A1*, *GCLC*, *GCLM*, *ARHGAP26*, *SCEL*, *IL-24*, *EIF5*, *NDRG1*, *SULT1A1*, *TXNRD1*, *CDC42EP2*, *NEBL*. qPCR was carried out using iTaq™ Universal SYBR® Green Supermix (BioRad). For *TIPARP*, *CYP1A1*, *CYP1B1* genes validation we used TaqMan® Gene Expression assay and TaqMan® Universal Master Mix (Applied Biosystem, Foster City, CA). The expression of selected genes was normalized against *GAPDH* (reference gene). For validation of miR-1909*, miR-29c, miR-1246, miR-146, RNU48, we used TaqMan microRNA assays (Applied Biosystem). The expression of selected genes was normalized against *RNU48* (reference miRNA). For the selected genes and miRNAs, fold changes values were calculated using the comparative CT method. Each test was carried out according the manufacturer's instructions. The details of qPCR assays are reported in Supplementary data, Table S1.

2.9. Analysis of multiple inflammatory proteins by antibody array

Antibody arrays Human Cytokine C1000 were purchased from RayBiotech. This antibody array was selected as representative of several inflammation signalling pathways and to screen the regulation of the complex interplays of the different proteins involved in the pro-inflammatory process. Briefly, after 24 h of exposure, multiwells with cells were stored at −80 °C overnight; cells were then homogenized on ice in provided lysis buffer using a syringe with needle and centrifuged (5 min, 10,000 rpm at 4 °C). Total amount of proteins was assessed by Bicinchoninic acid assay kit (Sigma Aldrich) and membrane arrays were performed according to the manufacturer's instructions; briefly, 400 μg of total proteins of each sample were pipetted onto different membranes and incubated for 2 h at room temperature (RT). The signal intensities for each antigen-specific antibody spots, proportional to the relative concentration of the antigen in the sample, were detected by chemiluminescence and recorded by a luminescence reader (Biospectrum-UVP) and densitometry analysis was then performed with dedicated software (VisionWorks LS-UVP). Data were expressed as signal intensity corrected by the signals of positive and negative controls following manufacturer's guidelines, and fold increase (FI) of PM exposed samples over unexposed (control) are reported in false colour scale.

2.10. Analysis of secreted inflammatory biomarkers by ELISA

After 24 h of exposure to the different treatments, the supernatants were collected to determine IL-6, IL-8, IL-1β, IL-24 and ICAM-1 protein levels by sandwich ELISA, according to the manufacturer's guidelines (Invitrogen or Abcam). The absorbance of each sample was measured by Multiplate Reader Ascent (Thermo) at 450 nm and 630 nm. Moreover, to test a possible adsorption of cytokines on particles, a cell-free system was set up in which IL-24 standards at a concentration of 500 or 1000 pg/ml and particles at 10 μg/cm^2 were incubated for 24 h in cell medium with 5% CO_2 at 37 °C. Supernatants were collected, centrifuged to remove particles, and processed by ELISA to determine the amount of free cytokine.

2.11. Western blotting

After 24 h of treatment cells were scraped from petri dishes and lysed on ice in RIPA buffer (150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 0.1% of protease inhibitors, added just before use). The total proteins content was evaluated by the Bicinchoninic Acid Protein assay kit (Sigma Aldrich) according to the manufacturer's instruction. Equal amounts of proteins were loaded, separated by 12% SDS-PAGE, and finally transferred on nitrocellulose membranes. Equal loading was assessed by red Ponceau staining of membranes. The membranes were then blocked for 1 h (TBS + 0.1% Tween20 + 5% (w/v) BSA or milk) and incubated at 4 °C overnight with specific primary

antibody diluted according to datasheets (NF-κB p50 and p105, phospho–NF–κB, IκB-α, phospho-IκB-α, HO-1, NQO1, HSP27, phospho-HSP27). After incubation for 2 h RT with the specific HRP-linked secondary antibodies (anti-rabbit IgG or anti-mouse IgG, Cell Signalling Technology), membranes were incubated with Chemi-luminescent Peroxidase Substrate (Sigma Aldrich). Digital images were taken by a luminescence reader (Biospectrum-UVP) and densitometry analysis performed with dedicated software (VisionWorks LS).

2.12. Flow cytometry evaluation of intracellular ROS

The quantitative measurement of intracellular reactive oxygen species (ROS) after 2 or 24 h of PM exposure was investigated by flow cytometry using H_2DCFDA-fluorescence, according to Gualtieri et al. (2012). Briefly, cells were incubated at 37 °C with H_2DCFDA (5 μM) in PBS for 20 min, then washed in PBS and treated with PM; after treatment, the cells were harvested, suspended in PBS, and the ROS-induced fluorescence was quantified at 525 nm. The results were normalized to the auto-fluorescence of cells and PM, assessed from negative controls (samples not stained with H_2DCFDA).

2.13. Evaluation of cytoskeleton organization by immunocytochemistry

Actin organization was analyzed by fluorescence microscopy following common techniques. Briefly, cells plated 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 RT. Cells were then stained with 100 nM Rhodamine Phalloidin (Cytoskeleton, Inc.) in PBS at RT in the dark for 30 min, and the DNA was counterstained with DAPI. Slides were observed under a fluorescence microscope (AxioObserver, Zeiss Germany) and representative digital images were taken.

2.14. Data analysis

Mean and standard error of mean (SEM) of three independent experiments (ELISA, western blotting and ROS production) are reported. Statistical analyses were performed using Sigma Stat 3.1 software, using one-way ANOVA with Dunnett or Dunn's as a post hoc test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Modulation of global gene expression in BEAS-2B cells exposed to seasonal PM

Gene expression profiling was performed using GeneChip U133 Plus 2.0. The results showed that seasonal PM fractions had a significant effect on the gene expression in BEAS-2B cells (Supplementary data, Table S2). After removing unannotated and repeated entities, we computed a total of 441 DEGs for the wPM and 542 DEGs for the sPM exposure. Most of the DEGs were up-regulated: 425 genes for wPM and 521 for sPM group. The remaining genes were down-modulated (16 for wPM, 21 for sPM group). Comparing the two lists of DEGs, we found a common signature including 339 genes that were up-regulated and 5 genes down-regulated, approximately 80% of all DEGs for wPM group and 65% for sPM. When the common differentially expressed genes were carefully examined, we found that 8 genes (*CYP1A1*, *CYP1B1*, *IL-8*, *KRT6B*, *MMP1*, *SERPIN B2*, *IL-24*, *IL-1B*) displayed a FC > 8, among them *CYP* genes were most up-regulated by wPM exposure (Supplementary data, Table S2). Remaining common DEGs exerted a fold change ranging from 2 to 3 compared to unexposed BEAS-2B cells.

3.2. Biological process and pathways modulated

The functional enrichment of DEGs lists using ToppFun tool revealed that sPM and wPM affected a common set of pathways related to inflammatory response such as TNF signalling, NF-κB signalling, NOD-like receptor signalling, cytokine–cytokine receptor interaction (Fig. 1). The bioinformatic analysis evidenced an enrichment of signalling associated with cancer and transcriptional misregulation pathways (Supplementary data Table S3). Specifically sPM exposure of BEAS-2B cells significantly affected processes involved in JAK-STAT signalling and lysine degradation, while wPM exposure peculiarly affected genes involved in the xenobiotic response processes, activation of glutathione biosynthesis (*GCLC*, *GCLM*), steroid hormone biosynthesis (*UGT1A1*, *AKR1C3*, *CYP1A1*, *CYP1B1*, *AKR1C1*, *AKR1C2*, *SULT1E1*) and small cell lung cancer (*PIK3R1*, *BIRC3*, *XIAP*, *CCND1*, *ITGA2*, *PTGS2*, *CYCS*, *NFKBIA*).

3.3. Gene expression validation by qPCR

To validate gene expression changes observed in the microarray analysis, qPCR of selected genes was performed on BEAS-2B cells

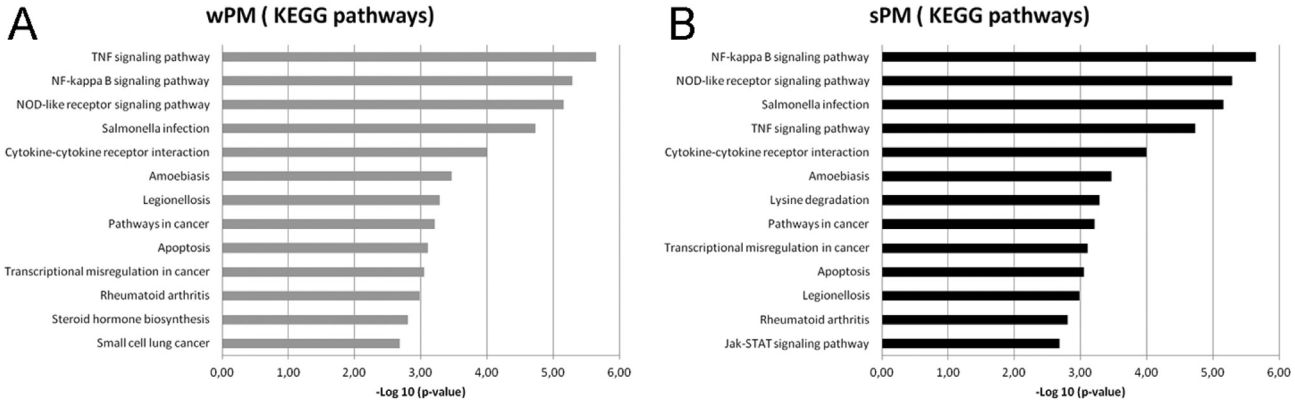


Fig. 1. Pathways analysis of up-regulated gene by microarray in wPM (A) and sPM (B) treated BEAS-2B cells. The enrichments data for KEGG pathways were obtained by using ToppFun tool (original data are reported in Supplementary Table S2). Bars represent the −log (p-value).

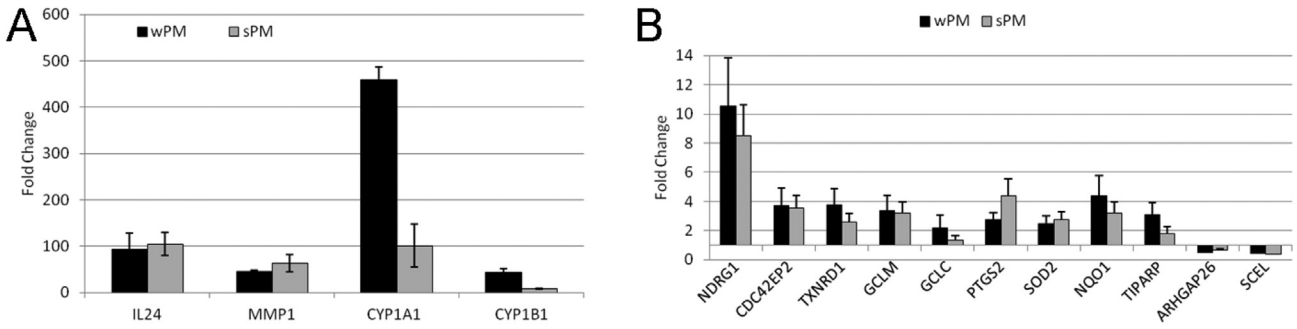


Fig. 2. qPCR validation of the selected DEG genes. (A) Fold change of very high up-regulated gene (FC > 20; see Supplementary table S2) and (B) fold change of medium up-regulated gene (see Supplementary table S2) are reported. Ct-values were normalized against housekeeping gene GAPDH. Fold changes were calculated as $2^{-\Delta\Delta Ct}$. Bars represent mean ± SD of separate experiments (n = 3).

exposed to sPM and wPM. Genes for validation were selected from those pathways mainly involved in PM-response, inflammation and oxidative stress. Accordingly to our previous results (Gualtieri et al., 2012), genes supposed to be involved in cytoskeleton alteration/reorganization (*MMP1*, *ARHGAP26*, *NDRG1*, *TXNRD1* and *CDC42EP2*) were further investigated. The qPCR results of 15 differentially modulated genes (13 up-regulated and 2 down-regulated) are reported in Fig. 2. Among the selected genes a strong up-modulation after both PM treatments for *CYP1A1*, *CYP1B1*, *MMP1* and *IL24* were evident, displaying a FC > 10. *CYP1A1* was most modulated by wPM (Fig. 2A). Moreover we validated the moderate up-modulation (range from 2 to 4 FC) for *CDC42EP2*, *TXNRD1*, *GCLM*, *SOD2* (Fig. 2B). The genes *GCLM*, *NQO1* and *TIPARP* were more up-modulated by wPM compared to sPM. Two genes (*ARHGAP26* and *SCEL*) were validated as down-regulated by qPCR.

3.4. Modulation of microRNAs and in silico analysis

By mean of a GeneChip miRNA 1,0 containing 1100 human microRNA (hsa-miR) we assessed the modulation of microRNAs at genome wide level. We found 12 miRNA that were modulated at significant threshold (P-value <0.05, FC[2]) by wPM or sPM treatment of BEAS compared to unexposed cells (Supplementary data, Table S4). MiR-1246 was found as the most up-regulated by sPM exposure. Two miRNA were found down regulated (miR-494 and miR-3201) by both PMs. The fold change of remaining miRNA was ranging from 2 to 4 fold change. Because miRNA may target more than 100 genes, the analysis was focused on their validated target genes using MiRwalk database and we intersected the list of validated target genes of with the lists of DEG (Supplementary data, Table S5). For miR-146a, miR-196 validated target genes were found (*data not shown*), which are involved in different processes such as defence response, cellular response to organic substance and regulation of immune system; among them 11 DEGs were modulated by sPM and resulted to be associated to inflammation signalling (*ADAM8*, *IL-1A*, *IL-1B*, *IL-6*, *IL-8*, *IRAK2*, *NLRP3*, *PLAU*, *PTGS2*, *SERPINE1*, *SOCS3*). For miR-29c, 157 validated target genes were extracted and they are implicated in cancer development (*data not shown*). Among them four DEGs were modulated by both PM (*LRRFIP1*, *PTGS2*, *CCND1*, *SPEN*, *TFPI2*) and two genes only by sPM (*CDK6*, *DICER1*). For miR-1246, miR-1184 and miR-1909* (miR-1909-5p) we found few validate target genes, only *DICER1* was included in our DEG list. Interestingly we found that down-modulated miR-494 has 31 validated target genes among them three DEGs were modulated by both PM (*LIF*, *LIFR*, *TOP2A*) and three DEG up-modulated by sPM (*CDK6*, *ADAM8*, *DICER1*). No data are available for miR-3201. Using MiRandola database, we found that three miRNAs -miR-146a, miR-1246 and miR-29- have been identified as circulating in plasma in pathologic condition or as circulating exosome related and Ago2 related (*data not shown*). Finally, according the HDMM database, we found many evidences indicating an implication of miR-146a, miR-29c in many human diseases such as cancers, cardiovascular and neurodegenerative disorder *(data not shown*). The documented role of miR-1246 in cancer or other disease is limited.

3.5. Validation by qPCR of selected up-regulated miRNA

The qPCR validation of five up-regulated miRNAs indicates that: miR-146a resulted up-regulated by both PM, but mainly after sPM exposure (FC of 92.24 compared to untreated cell); miR-1246 was up-regulated by both PM at similar levels of FC; both miR-29c and miR-1909* (miR-1909-5p) were up-regulated only in wPM treated cells (Table 1). For miR-1184 a reliable computation of the fold of expression was not possible because the Ct value were higher than 35 (*data not shown*).

3.6. Pro-inflammatory response pathway activation

Since gene and miRNA expression data highlighted the activation of pathways related to inflammatory response (TNF signalling, NF-κB signalling, NOD-like receptor signalling, cytokine–cytokine receptor interaction) in PM-exposed cells, their activation at protein level was investigated. Intracellular proteins expression was evaluated by antibody array and the activation of inflammatory pathways was confirmed, especially in cells exposed to sPM, as reported in Fig. 3. The data confirmed the modulation of several inflammatory proteins; significantly sPM induced higher modulation of inflammatory proteins (ENA78, IL8, IL1β, GRO, MIP3α, IGF1) in agreement with the higher upregulation of related genes (CXCL5, IL8, IL1β, CXCL1, CCL20, IGFBP3) in comparison to wPM.

Among genes and proteins modulated by PM, the release into the extracellular medium of pro-inflammatory interleukins (IL-6, IL-8, IL-24 and IL-1β) and the Intercellular Adhesion Molecule 1 (ICAM1), involved in the signalling inflammatory pathway of circulating leukocytes to the vascular endothelium, has been measured by ELISA. The results (Fig. 4A) showed a great release of IL-8 after sPM treatment (580 pg/ml in comparison with 292 pg/ml in control cells), while a significant decrease was detected after wPM treatment (180 pg/ml). A lower amount of IL-6, significant only for sPM (400 pg/ml) and not for wPM treatment (100 pg/ml) in comparison with control cells (60 pg/ml) was detected. Despite at gene level was evident a high fold increase of IL-24 in cells exposed to PM (Supplementary data Table S2 and Fig. 2), at protein level a low but significant increase of this interleukin (90 pg/ml with sPM and 80 pg/ml with wPM treatment, in comparison with 35 pg/ml in

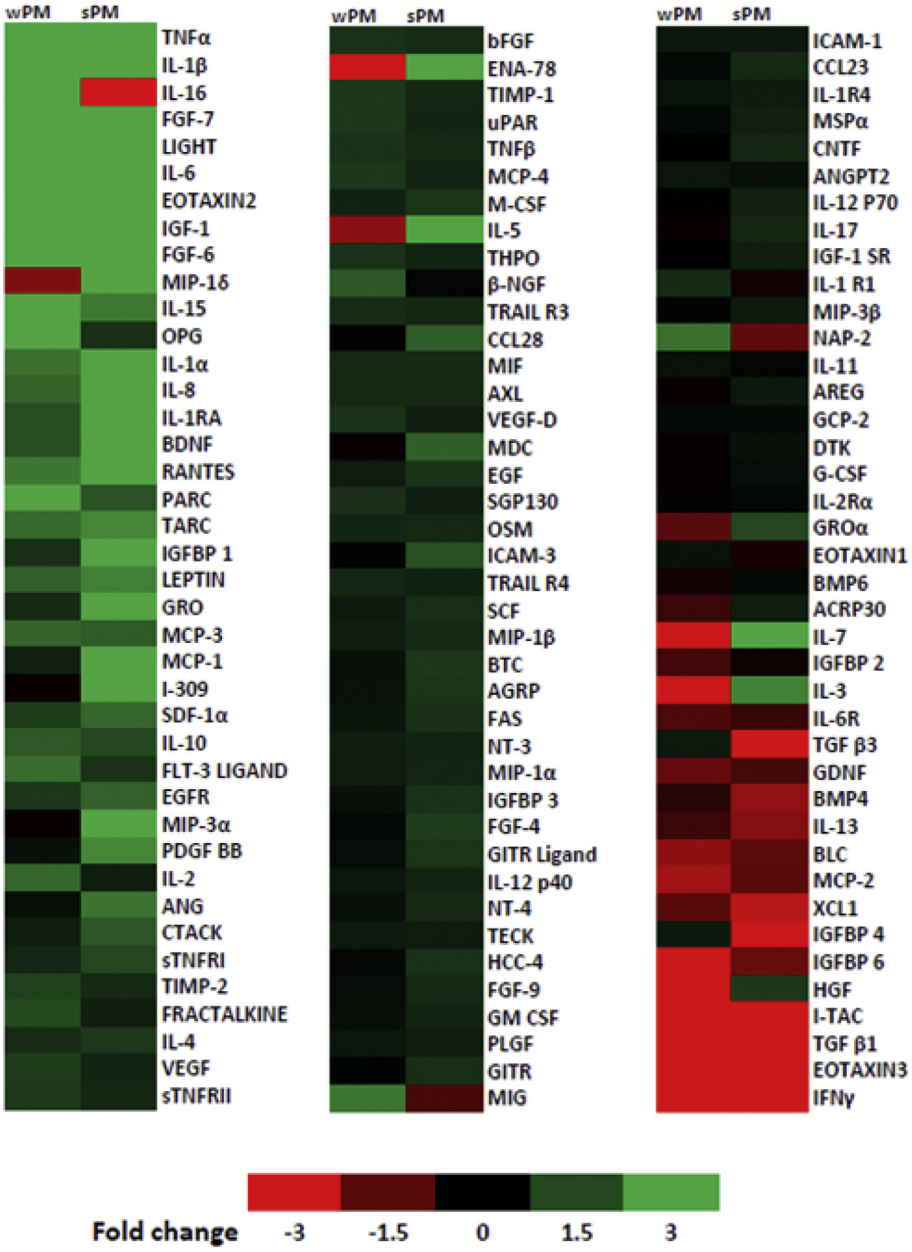


Fig. 3. Intracellular expression of proteins involved in inflammatory responses, assessed by membrane protein array in BEAS-2B cells exposed for 24 h to different PM. Fold changes of protein expression, increased (green), decreased (red) or equal (black) compared to untreated cells, are reported (n = 2).

control cells, Fig. 4A) was detected. Interestingly, the experiments performed in cell-free condition showed that IL-24 was adsorbed onto PM particles, suggesting that the amount of cytokines in our results could be underestimated (at least of 20%) (*data not shown*). Modulation of ICAM1 gene is associated only to wPM treatment, with a FC of 2.2; however the release of this protein was not significant, though a slight increase is apparent in sPM exposed cells (*data not shown*). Similarly IL-1β release was not affected by PM exposure, even if at gene level we found a FC of 7.2 and 8.2 after wPM and sPM exposure respectively (*data not shown*); this result suggests that the complex mechanism leading to release of this protein is not completely fulfilled.

Many proteins actively participate in inflammatory responses, but the mainstream activation of NF-κB pathway is crucial; therefore the levels of this protein, its phosphorylated form, and its inhibitor binding molecule (IκB-α) were evaluated with western blot to assess its involvement in biological responses. The data demonstrated that IκB-α significantly decreased after both treatments, with consequent NF-κB activation (Fig. 4B) confirmed by a 1.7-fold increase of phospho- NF-κB (activated form) and a simultaneous increase of NF-κB p50 form, which can translocate into the nucleus and activate target genes. The phosphorylated form of IκB-α was also analysed, but no significant modulation was apparent after 24 h of treatments, and it may depend by early proteasomal degradation (*data not shown*).

3.7. Oxidative stress pathway activation

The overexpression of proteins with oxidoreductase or antioxidant activity (NQO1 and HO-1) and the formation of ROS have been

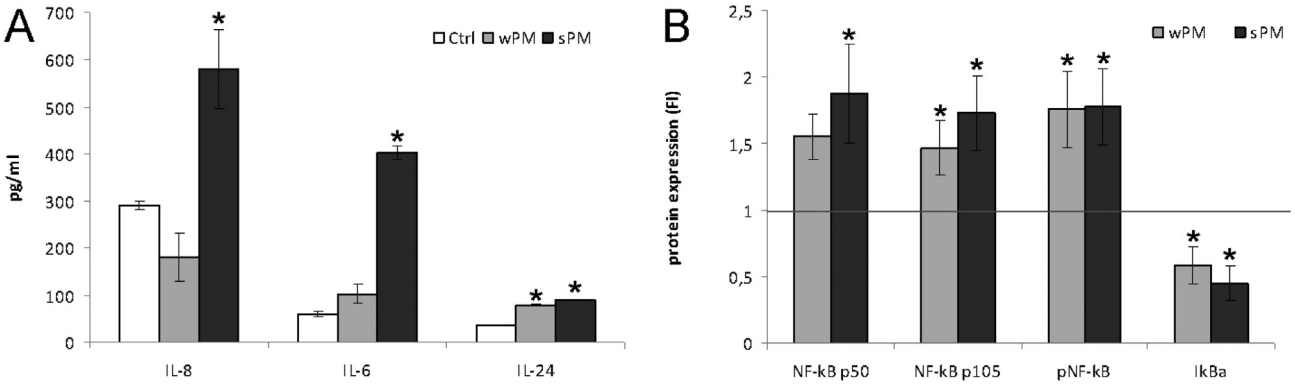


Fig. 4. Inflammatory pathway activation in BEAS-2B cells exposed to PM for 24 h. (A) IL-6, IL-8 and IL-24 release assessed by ELISA. (B) NF-κB and IκB-α modulation determined by western blotting. Bars represent mean ± SEM of separate experiments ($n \geq 3$). *$p < 0.05$ compared to untreated cells. Line equal to 1 represents control value.

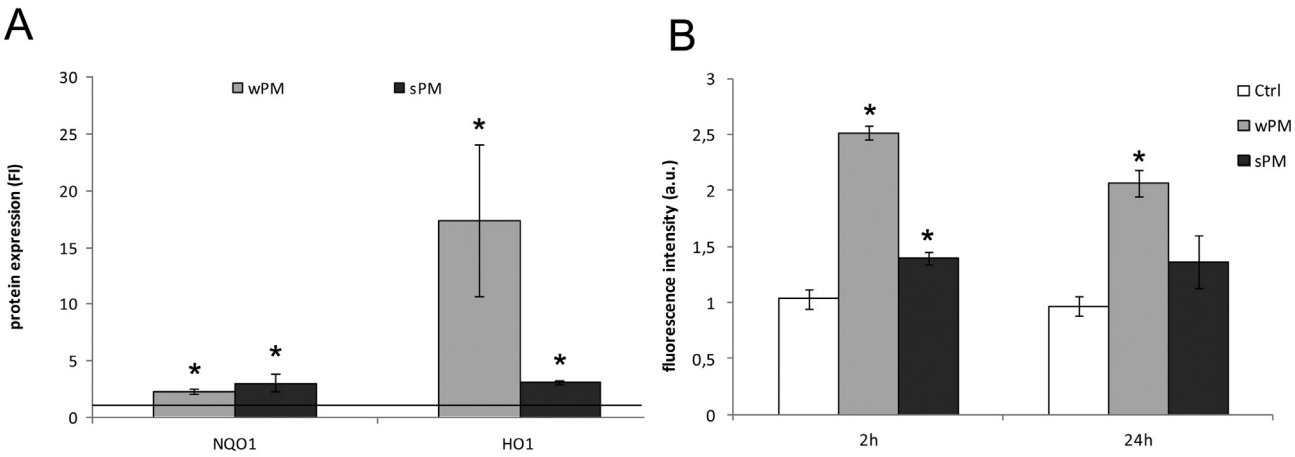


Fig. 5. Validation of oxidative stress pathway activation in BEAS-2B cells exposed to PM. (A) protein expression after 24 h of exposure. (B) ROS production, reported as fluorescence intensity, evidenced by flow cytometry analysis after 2 and 24 h of PM exposure. The data represent the mean + SEM of 3 experiments (ANOVA, Dunnett's). Bars represent mean ± SEM of separate experiments ($n \geq 3$). *$p < 0.05$ compared to untreated cells.

analyzed in order to validate gene and miRNA results, reporting the modulation of pathways involved in oxidative stress (xenobiotic response processes, activation of glutathione biosynthesis) in PM-exposed cells.

Western blot data (Fig. 5A) confirmed a significant 2.3-fold increase of NQO1 after wPM exposure and a 3-fold after sPM; HO-1 levels were especially augmented by wPM with 17-fold, while sPM increased it by 3-fold. Significant ROS formation was observed in BEAS-2B cells exposed to wPM for 2 or 24 h, with 2.5 and 1.3-fold respectively, while sPM induced minor increase of ROS, which was significant (2.1-fold increase) only at 2 h of exposure (Fig. 5B).

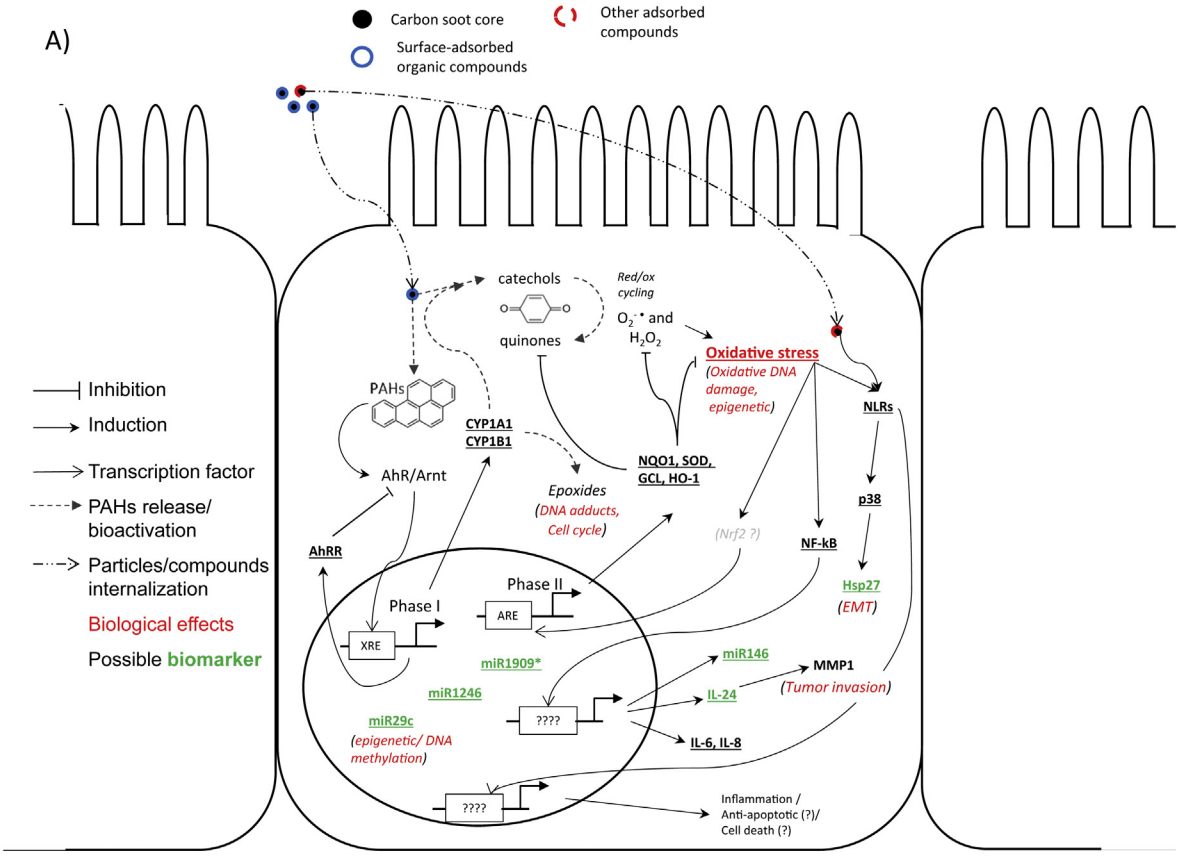
3.8. Cytoskeleton organization

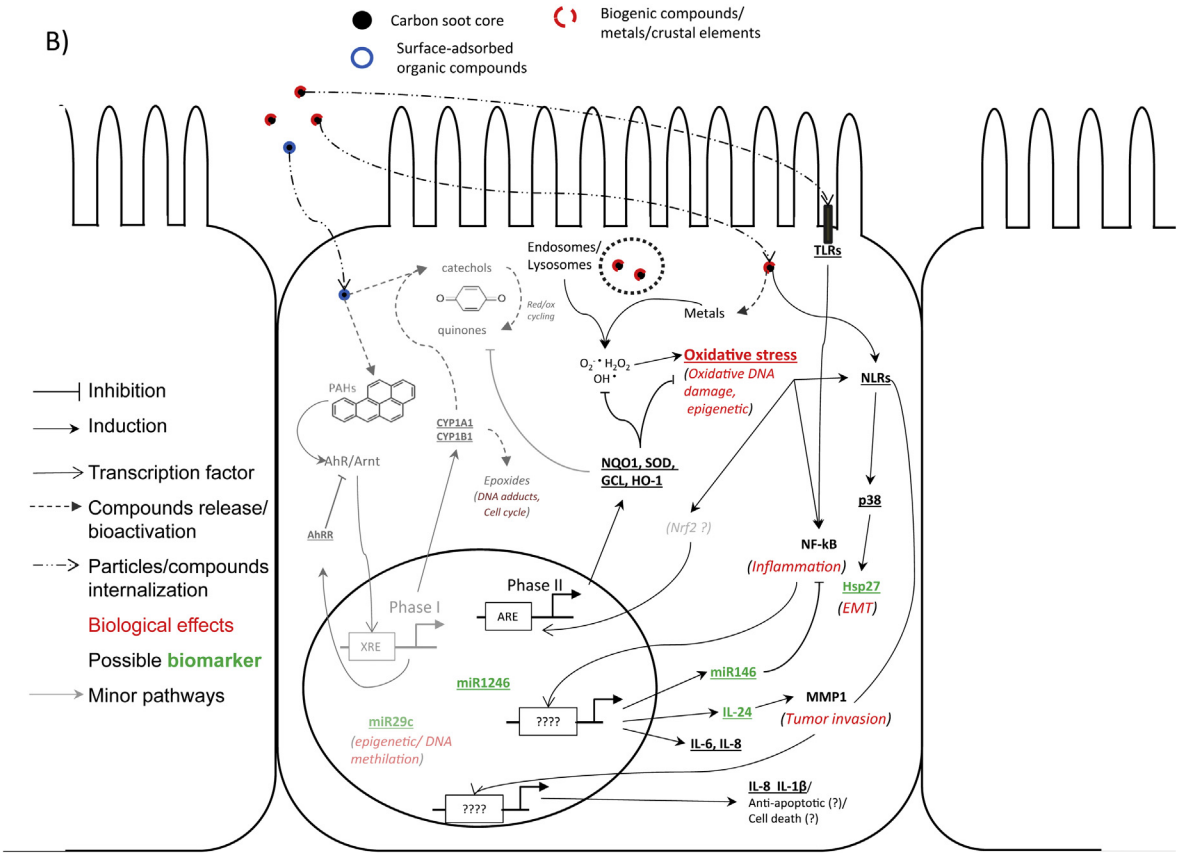
Since genes involved in cytoskeleton organization and actin/tubulin polymerization were modulated (Supplementary data Figure S1), the cytoskeleton was analyzed by phalloidin, which binds to filamentous actin (F-actin). Untreated cells showed a uniform distribution of actin filaments in the cytosol and near the cell periphery expressed in a form of stress fibers (Supplementary data Figure S1A) cells grown in sub-confluent areas exhibited typical fine actin projections. Cells exposed to PM presented membrane ruffles with an incomplete contact between adjacent cells (arrows). Since HSP27 activity regulates cytoskeleton organization (Mizutani et al., 2010), the expression of this actin-binding protein and its phosphorylated forms was evaluated by western blot analysis. A significant and similar decrease of HSP27 after 24 h of both treatments, with a simultaneous increase of phospho-HSP27 was apparent (Supplementary data Figure S1B).

4. Discussion

A transcriptomic profiling by microarray technology has been here used as a powerful tool to rapidly investigate the entire genome for the individuation of genes, miRNA and related pathways modulated in BEAS-2B cells exposed to sPM and wPM. In order to validate these data we have integrated the results with the evaluation of selected pathways activated at protein level. The molecular networks activated by the exposure to the different PMs, even if not completely understood, have evidenced interplay among oxidative stress, inflammation and DNA damage responses. The activation of these pathways determines DNA transcription and "de novo" protein synthesis. Several miRNA were differentially expressed after PM treatments and their role as possible biomarker of exposure can be hypothesized. The molecular signature, identified in cells exposed to sPM and wPM, is here reported in a tentative model (Fig. 6).

Systemic inflammation has been suggested to be a critical step in PM-induced health effects and it has been related to health effects after acute short-term PM exposures (Brunekreef, 2005;


A)
Carbon soot core
Surface-adsorbed organic compounds
Other adsorbed compounds
Inhibition
Induction
Transcription factor
PAHs release/ bioactivation
Particles/compounds internalization
Biological effects
Possible biomarker
catechols
quinones
Red/ox cycling
O2•- and H2O2
Oxidative stress
(Oxidative DNA damage, epigenetic)
PAHs
CYP1A1
CYP1B1
AhR/Arnt
AhRR
NQO1, SOD, GCL, HO-1
Epoxides
(DNA adducts, Cell cycle)
(Nrf2 ?)
NLRs
p38
NF-kB
Hsp27
(EMT)
Phase I
Phase II
XRE
ARE
miR1909*
miR146
MMP1
(Tumor invasion)
miR1246
IL-24
miR29c
(epigenetic/ DNA methylation)
????
IL-6, IL-8
Inflammation / Anti-apoptotic (?)/ Cell death (?)


B)
Carbon soot core
Surface-adsorbed organic compounds
Biogenic compounds/ metals/crustal elements
Inhibition
Induction
Transcription factor
Compounds release/ bioactivation
Particles/compounds internalization
Biological effects
Possible biomarker
Minor pathways
Endosomes/ Lysosomes
TLRs
catechols
quinones
Red/ox cycling
Metals
O2•- H2O2 OH•
Oxidative stress
(Oxidative DNA damage, epigenetic)
NLRs
PAHs
CYP1A1
CYP1B1
AhR/Arnt
AhRR
Epoxides
(DNA adducts, Cell cycle)
NQO1, SOD, GCL, HO-1
(Nrf2 ?)
p38
NF-kB
(Inflammation)
Hsp27
(EMT)
Phase I
Phase II
XRE
ARE
miR1246
miR146
MMP1
(Tumor invasion)
IL-24
miR29c
(epigenetic/ DNA methilation)
????
IL-6, IL-8
IL-8 IL-1β/
Anti-apoptotic (?)/
Cell death (?)

Motta et al., 2013). Proteins as TNF-α, IL-1β, IL-6 and IL-8 have been already linked to PM-induced pulmonary inflammatory processes and systemic effects i.e. blood clot formation and endothelial dysfunction (Alfaro-Moreno et al., 2008). Although both tested PM affected the modulation of these genes and other involved in inflammatory pathways, such as TNF signalling, NF-κB signalling, NOD-like receptor signalling and cytokine–cytokine receptor interaction, our results demonstrate that pro-inflammatory response at protein level is induced only by sPM (Fig. 6B). In fact, the biochemical evaluation of proteins expression shows that both PM (summer at a higher extent) increase the intracellular level of IL-6 and IL-8, but only sPM is able to induce their release in the extracellular medium. These observations agree with the epidemiological data relating PM exposure with asthma and chronic obstructive pulmonary disease (COPD) (Brunekreef, 2005). A strict correlation between respiratory disease hospitalization and coarse PM concentration has been reported during summer. In Northern Italy too hospital admissions for respiratory or cardiovascular causes have been found to be increased during summer (Conti et al., 2015), although the higher concentration of PM are reported in winter. This event is partly related to particulates chemical composition since only sPM induces IL-1β release in exposed macrophages, as a consequence of the activation of membrane TLRs, ROS formation and activation of the inflammasome NLPR3 by the biological and inorganic components of sPM (Bengalli et al., 2013).

However beside these processes, other mechanisms are crucial for inflammation; NF-κB is a redox-related transcription factor that activates a pro-inflammatory response to ROS-induced oxidative stress. Here we report radical species formation and NF-kB activation after cells exposure to sPM and wPM too (Fig. 6A). The low inflammatory potential of wPM may depend on the presence of a high content of organic compounds. Øvrevik et al., 2010; Øvrevik et al. (2014) have reported that PAHs and other hydrocarbons may have a different impact on the pro-inflammatory response for a specific activation of the aryl hydrocarbon receptor (AhR); moreover Baglole et al. (2008) demonstrated that AhR activation is responsible for the limited inflammation in lung fibroblasts exposed to cigarette smoke. *CYP1A1* and *CYP1B1* are AhR principal responsive genes and they encode for enzymes involved in PAHs, quinones and dioxins metabolism, and AhRR which represses AhR function through a negative feedback loop. However increasing evidences suggest that AhR also plays an important role in the regulation of several cytokine and chemokine genes, competing with NF-κB subunit RelA for common co-factors (Haarmann-Stemmann et al., 2009), suppressing p65, and through other pathways still poorly understood (Øvrevik et al., 2014). On the other hand, combined exposure to tumour necrosis factors (TNF) or mixture of cytokines and PAHs may also lead to upregulation of CYP1B1 and increased DNA damage (Smerdová et al., 2014). We have previously reported (Gualtieri et al., 2011) a significant modulation of *AHRR* and *CYP* genes, and CYP proteins in cells exposed to wPM and this evidence is here confirmed (Fig. 6A). In agreement with the mechanism for PAHs bio-activation (Xue and Warshawsky, 2005), the modulation of these genes and proteins is related to ROS formation, DNA damage and cell cycle alteration (Longhin et al., 2013; Gualtieri et al., 2011, 2012). The possible inhibitory effect of this pathway on the inflammatory response of Milan wPM2.5-exposed cells deserves further investigation.

The ability of sPM and wPM to induce inflammation may also depend on their oxidative potential. Harijith et al. (2014) reported that silicosis and asbestosis origin from endocytosis of inhaled silica and asbestos particles by lung macrophages, and this determines increased ROS generation, lysosome destabilization and NLRP3 inflammasome activation. We have previously demonstrated (Bengalli et al., 2013) that ROS increase participates to inflammasome activation and IL-1β release in macrophages exposed to sPM. Here ROS formation and the activation of genes related to antioxidant pathways in cells exposed to both PM are described (Fig. 6), even if wPM modulates *NQO1*, *GCLC* and *TIPARP* genes and related proteins in a stronger manner. Moreover heme-oxygenase 1 (HO-1) protein levels are heavily increased and these data suggest that antioxidant and xenobiotic response genes interplay to protect cells against oxidant or pro-oxidant insults (Nioi and Hayes, 2004).

Despite their deep differences, both particles have serious effects on exposed cells. While short-term PM exposure is related to acute inflammation producing respiratory and cardiovascular outcomes (Kim et al., 2015), chronic inflammation is known to be involved in tumorigenesis (Hasselbalch, 2013; Sever and Brugge, 2015). Also the effects produced by wPM exposure, i.e. DNA damage and cell cycle alteration (Longhin et al., 2013), have been associated to chromosomal instability and a consequent cancer development (Fukasawa, 2005; Tian et al., 2015). In our experiments both sPM and wPM modulate the expression of genes and proteins involved in cancer development, extracellular matrix, cytoskeleton organization and inflammation e.g. MMP1, HSP27, IL-24 (Fig. 6). These results may be the basis for the individuation of common exposure biomarkers appropriate for both PM, even if responsible of different molecular effects induction. A possible biomarker is the matrix metalloproteinase 1 (MMP1), overexpressed in cells exposed to both PM. MMP1 is up-regulated in a wide variety of cancers and Sauter et al. (2008) suggested its association with tumor invasion and metastasis. This protein is increased after exposure to tobacco smoke and induced an increase in collagenase activity with a consequent decrease of cell adhesion and easier penetration of particles (Sauter et al., 2008). Recently Montaño et al. (2014) has indicated MMP1 as biomarkers of COPD associated to biomass and tobacco smoke. Thus enhanced levels of MMP1 may be a marker of increased risk for lung cancer and its increase has been associated both to AhR activation (Feng et al., 2013) and to IL-1β signaling (Klatt et al., 2006). Both wPM and sPM may activate extracellular matrix (ECM) remodeling genes through distinct pathways even promoting different outcomes. Therefore PM ability to activate ECM genes may be a possible marker of exposure to particulates, whatever is their chemical composition.

In this respect, another possible biomarker for PM-induced cancer transformation is HSP27, downstream effector of p38 MAPK, that is active in regulation of cytoskeleton organization, inhibition of apoptosis and induction of epithelial to mesenchymal

Table 1
Comparisons of miRNA expression data between microarray and QPCR validation analysis.

miRNA	wPM (fold change)*		sPM (fold change)*	
	microarray	qPCR	microarray	qPCR
Hsa-mir-1246	2.14	7.47	11.17	8.59
Hsa-miR-146a	1.42	11.13	3.69	92.24
Hsa-miR-1909*	3.16	3.10	3.45	0.54
Hsa-miR-29c	2.79	2.45	2.33	1.67

*Average of fold change of exposed cells compared to untreated controls (n > 3).

Fig. 6. Conceptual models of the molecular signatures identified in cells exposed to winter (A) and summer (B) PM is reported. The main cellular pathways activated by PM exposure are reported in relation to specific chemical properties. Proteins significantly activated (in bold) and protein related to secondary pathway or presumed to be activated (in grey) are listed. Tentative marker of exposure/effects related to human health disease also identified (in green).

transition (EMT) (Mizutani et al., 2010). The activation of HSP27 after exposure to sPM and wPM, here reported as an increase of the phosphorylated form, may explain the morphological modification of BEAS-2B cells. High levels of HSP27 have been found in numerous human tumor cells and its phosphorylated form is gaining acceptance as a biomarker of cancer and cardiovascular disease (Vidyasagar et al., 2012). Interestingly, this protein has been found to have a role in PM-induced endothelial barrier disruption after ROS formation and p38 activation (Wang et al., 2010b) and its level in plasma and lymphocytes has been suggested as possible biomarker for lung cancer induced by occupational coal-mine dust exposure (Wang et al., 2010a). Also the genes integrin *ITGA2*, *CDC42* and *ARHGAP26*, related to cytoskeleton organization and actin/tubulin polymerization, are modulated and they may be responsible for changes in cell morphology, such as the formation of membrane ruffles and protrusive structures (filopodia) (Supplementary data Figure S1A). These modifications have been related to EMT (Yilmaz and Christofori, 2009); in particular filopodia inducing genes have been related to cancer progression, while altered expression of integrins has been related with poor prognosis in human cancer (Arjonen et al., 2011).

An increase of IL-24 at mRNA and protein levels in cells exposed to both PM has been observed in our experiments. This protein induces JAK1-STAT3 and MAPK pathways, leading to the release of IL-8 and MMP1 (Jin et al., 2014) and it has also been suggested to be a killing cytokine, selectively targeting tumour stem/initiating cells for death by apoptosis, and consequently considered a target for cancer treatment (Menezes et al., 2014). Up-regulation of IL24 and other oncogenic and immune-modulated genes is induced by low-dose of cadmium exposure in normal prostate epithelial cells (Bakshi et al., 2008). Recently the production of IL-24 has been reported to be stimulated by environmental toxic stressors, such as chemical irritants and ultraviolet irradiation, in human epidermal keratinocytes NHKs (Jin et al., 2014) but to our knowledge the expression of this cytokine associated to airborne particles exposure is here described for the first time. Thus the role of this cytokine as biomarker of PM exposure needs further investigation.

MiRNAs have been indicated as possible modulators for several biological pathways (Hou et al., 2011) and here miR-146a and miR-1246 resulted up-regulated by PM exposure (Table 1 and Fig. 6B). Changes in mir-146a expression have been observed in human diseases (Li et al., 2010) and it has been suggested to play an important role in the regulation of innate immune and inflammatory response (Saba et al., 2014). Interestingly, there are several binding sites for NF-kB in the promoter region of mir-146a, and the induced expression of this miRNA by LPS, IL-1β and TNF-α is NF-kB dependent. The transcription factor NF-kB has been heavily indicated as a causal link between inflammation and carcinogenesis (Williams et al., 2008) and recently mir-146a has been described in the blood of foundry workers exposed to PM metal-rich (Motta et al., 2013) and in tobacco smoke exposure (Zago et al., 2014). The specific increase of this miRNA supports its role as possible molecular biomarker for PM-induced lung inflammation and asthma (Comer et al., 2014). MiR-1246 has been reported to be a target of the p53 family, and inhibits Down syndrome-associated DYRK1A, consequently activating NFAT1c and inducing apoptosis (Zhang et al., 2011). Takeshita et al. (2013) have showed that miR-1246 expression was up-regulated in colorectal cancer. At present whether miR-1246 acts as an oncomir or tumour suppressor is unknown. MiR-29c, in our experiments significantly up-regulated by exposure to wPM (Table 1 and Fig. 6A), is involved in non-small cell lung cancer (Zhu et al., 2014) and its implication in epigenetic modification has been suggested (Fabbri et al., 2007). Our results confirm the recent evidences (Hou et al., 2011; Loomis et al., 2013) reporting that some miRNAs are involved in biological responses to air pollutants and they may have a potential role as biomarkers of PM exposure (Vrijens et al., 2015).

5. Conclusion

In summary, we propose a possible integrated molecular signature including genes, miRNA and proteins for PM exposed BEAS2-B cells. Nonetheless further investigations are needed to clarify the health impact of genetic and epigenetic modification induced by PM exposure.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.11.013.

Competing financial interests

Authors declare they have no actual or potential competing financial interests.

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