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Extracellular vesicle-driven information mediates the long-term effects of particulate matter exposure on coagulation and inflammation pathways.

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Abstract

Background—Continuous exposure to particulate air pollution (PM) is a serious worldwide threat to public health as it coherently links with increased morbidity and mortality of cardiorespiratory diseases (CRD), and of type 2 diabetes (T2D). Extracellular vesicles (EVs) are circular plasma membrane fragments released from human cells that transfer microRNAs between tissues. In the present work it was explored the hypothesis that EVs with their encapsulated microRNAs (EVmiRNAs) contents might mediate PM effects by triggering key pathways in CRD and T2D.

Methods—Expression of EVmiRNAs analyzed by real-time PCR was correlated with oxidative stress, coagulation and inflammation markers, from healthy steel plant workers (n=55) with a well-characterized exposure to PM and PM-associated metals. All p-values were adjusted for multiple comparisons. In-silico Ingenuity Pathway Analysis (IPA) was performed to identify biological pathways regulated by PM-associated EVmiRNAs.

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Results—Increased expression in 17 EVmiRNAs is associated with PM and metal exposure ($p<0.01$). Mir-196b that tops the list, being related to 9 different metals, is fundamental in insulin biosynthesis, however three (miR-302b, miR-200c, miR-30d) out of these 17 EVmiRNAs are in turn also related to disruptions ($p<0.01$) in inflammatory and coagulation markers.

Conclusions—The study's findings support the hypothesis that adverse cardiovascular and metabolic effects stemming from inhalation exposures in particular to PM metallic component may be mediated by EVmiRNAs that target key factors in the inflammation, coagulation and glucose homeostasis pathways.

1. Introduction

Continuous exposure to particulate air pollution (PM) is a serious worldwide public health problem, PM is linked to increased morbidity and mortality, particularly due to cardiovascular, respiratory diseases (CDR) and even more recently, to type 2 Diabetes (T2D) (Pope et al. 2002, 2004; Brook et al. 2013; Wang et al. 2014).

Epidemiologic (Brook et al. 2004; Chen and Lippmann, 2009) and in vivo studies (Chen and Hwang 2005; Corey et al. 2006), provide evidence that the metallic particles might have a central role in mediating PM toxicity and might be responsible for such effects. Foundry work has also been associated with a number of adverse health outcomes, including cardiovascular and respiratory diseases (Andjelkovich et al. 1990; Kuo et al. 1999), which might be caused by high level of metal-rich PM exposure. In fact, even in modern foundry facilities that benefit from state-of-art measures for exposure reduction, the workers are still exposed to high levels of airborne metal-rich PM well above those found outdoors (Bergamaschi et al., 2005; Tarantini et al., 2009).

The mechanisms linking metal-rich PM exposure with CRD and T2D diseases have not yet been fully elucidated. Oxidative stress and inflammation, two shared mechanisms of these disorders, have been linked with long-term PM exposure (Mannucci, 2013; Esposito et al. 2016, Rajagopalan and Brokk, 2012). One main working hypothesis is that PM with an aerodynamic diameter of 2.5 μm or less (PM_{2.5}, PM₁) can deeply penetrate into the lung, deposit in the alveolar area, and locally trigger oxidative stress and inflammatory responses. Particularly metals, directly as catalysts for ROS production, and indirectly, through their metabolism, are even important sources for cellular oxidant generation (Brook et al., 2010). This pulmonary local oxidative-inflammatory reaction leads to a subsequent systemic inflammation (Brook et al., 2010) and to a worsening of insulin resistance index (Sun et al. 2009). Several blood inflammatory markers, including C-reactive protein (CRP) (Li et al. 2012), interleukin (IL)-6 (Kido et al. 2011), fibrinogen (Peters et al. 2009), platelet activation (Wilson et al. 2010) and impairment of glucose homeostasis (Eze et al. 2015) were all found altered in response to PM exposures. However, how the pulmonary and systemic compartments communicate with each other remains to be fully understood. This pulmonary local reaction might be followed by the release of pro-inflammatory signals able to reach the systemic circulation. Alongside the release of pro-inflammatory mediators, pulmonary cell-derived membrane vesicles may also be secreted, representing another emerging way of intercellular communication (Raposo and Stoorvogel 2013).

Extracellular vesicles (EVs) are circular plasma membrane fragments actively released from human cells (Colombo et al. 2014). The release of EVs are induced in response to physical and chemical stress, oxidative stress included (Ratajczak et al. 2006). EVs, after internalization within target cells possibly through surface-expressed ligands, can transfer microRNAs (miRNAs) from one cell to another (Hunter et al. 2008; Skog et al. 2008) enabling intercellular and inter-organ communication in the body (Hunter et al. 2008). One single miRNA can regulate hundreds of target mRNAs in interrelated gene pathways and a single mRNA can be targeted by several different miRNAs (Lewis et al. 2005). Plasma circulating miRNAs packed into EVs [EV-encapsulated microRNAs (EVmiRNAs)] are more stable than the circulating ones (Tsui et al. 2002). EVmiRNAs might be the ideal candidates to mediate the effects of PM, since potentially they could be produced by the respiratory system, (Prado et al. 2008; Kesimer et al. 2009) reach the systemic circulation (Orozco and Lewis 2010) and lead to the development of cardiovascular dysfunction (Puddu et al. 2010).

In our recent work, increased expression of miR-128 contained within plasma EVs associated both in vivo and in vitro to short-term metal-rich PM exposure, as it was modified after 3 days of workplace PM exposure (Bollati et al. 2015). In the present work the hypothesis that long-term occupational exposure to PM and PM-associated metals influences expression of EVmiRNAs has been explored, and that the elicited response is correlated, in turn, with long-term effect to PM exposure, i.e., inflammatory and coagulation disruptions. In order to address this hypothesis, the expression of 88 EVmiRNAs has been measured in 55 steel plant workers, well-characterized in their PM exposure, evaluated its association with PM exposure and then with markers of oxidative stress (i.e. 8-hydroxyguanine measured in blood mitochondrial DNA, 8-OH-dG), coagulation (i.e. t-PA antigen, D-dimer, ETP TM+, ETP TM-) and inflammation (i.e. C-reactive protein, CRP).

2. Materials and methods

2.1. Study participants.

We recruited 63 male healthy workers (mean age=44 years, range between 27 and 55 years) employed for at least one year in a steel production plant near Brescia, Northern Italy (Bonzini et al. 2010). Plasma was available for 55 workers. In order to analyze long-term effects of PM, we obtained blood samples for oxidative stress, coagulation and inflammation markers, at two different times: i) sample 1 was collected in the morning of the first day of a working week (following two days off work) before the beginning of any work activity; ii) sample 2 (post-exposure) was collected at the same time on the fourth work-day, following three consecutive days of work. Data from sample 1 and 2 were analyzed jointly, enhancing thus statistical power and avoiding potential masking of effects due to day-to-day variability, and then taken as a measure of long-term effects. The local Institutional Review Board (Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Milano) approved the study on April 4, 2010. An individual written informed consent was obtained from each participant. Baseline and post exposure samples were analyzed jointly in repeated measure analysis and taken as a measure of long-term effects (Tarantini et al. 2009).

2.2. PM exposure assessment.

Measures of airborne PM and PM metallic components obtained in each of the 11 work areas of the steel production facility were used to estimate individual exposure of each worker as previously described (Tarantini et al. 2009; Cantone et al. 2011). Briefly, measures of PM included levels of PM with aerodynamic diameters $10\text{ }\mu\text{m}$ (PM₁₀) and $1\text{ }\mu\text{m}$ (PM₁) that were detected using a GRIMM 1100 light-scattering dust analyzer (Grimm Technologies, Inc., Douglasville, GA, USA). Concentrations of coarse particles were calculated from these measures as the difference between PM₁₀ and PM₁. PM metal components in PM₁₀ fraction were detected through multielemental analysis by means of inductively coupled-plasma mass spectrometer (ELAN DRC II; PerkinElmer, Waltham, MA, USA). During the 3 working days, each of the study subjects recorded in a personal log the time spent in each of the work areas. Individual exposure was calculated as the average of PM weighted by the time spent in each area. PM levels in each of the work areas showed very little variability over time, as compared to measures repeated over 1 year showing a very high correlation between PM concentrations ($r^2 > 0.90$). Because all the study subjects reported in the questionnaire to have performed their standard work routine during the 3 days of the study, the time-weighted PM represented, in addition to the exposure during the week of the study, a measure of the usual exposure of the study subjects. Therefore, taking into consideration that study subjects were continuously exposed to the levels of PM measured during the week of the examination, the statistical analysis was done as specified below, including the evaluation of the association of PM (PM₁₀, PM₁, Coarse, Metals) levels with miRNA expression in circulating EVs and outcomes related to oxidative stress, coagulation and inflammation pathways, measured at the beginning (sample 1) and at the end of the work week (sample 2), which were analyzed jointly in repeated measure analysis and taken as a measure of long-term effects.

2.3. Blood collection, EV isolation, and RNA extraction.

Peripheral blood sample 1 and sample 2 (7 ml) were collected in EDTA tubes and centrifuged at $400 \times g$ for 15 minutes to separate plasma fraction. Subsequently, aliquots of cell-free plasma were stored at -80°C . Before use, all samples were kept on melting ice to allow thawing. After thawing, samples were first centrifuged three times at $800 \times g$ for 15 min at 4°C to remove cell debris, remnants and aggregates. The supernatant was then ultra-centrifuged at $110,000 \times g$ for 2 h at 4°C . The supernatant was removed and the MVs were re-suspended in 700 μl di QIAzol Lysis reagent (Qiagen). Enriched miRNAs were isolated by miRNeasy purification kit (Qiagen) following manufacturer instructions.

PAXgene blood RNA tubes (Qiagen) were also collected and PAXgene blood RNA kit (Qiagen) was used to extract total RNA. The PAXgene tube contains a reagent that immediately stabilizes intracellular RNA.

2.4. Coagulation tests and inflammation markers

Blood for coagulation testing was drawn into vacuum tubes (Becton Dickinson, Meylan, France) containing 0.109 M trisodium citrate at a ratio of 9:1 (blood/anticoagulant). Blood was centrifuged for 15 minutes at $2,880g$ at room (controlled) temperature and the supernatant plasma was harvested, aliquoted in plastic tubes, quickly frozen in liquid

nitrogen and stored at -80°C until testing. The thrombin generation assay, that measures the endogenous thrombin potential (ETP) was run as described by Bonzini et al. (2010). It was based on the activation of coagulation in platelet-poor plasma with calcium chloride, without exogenous triggers and performed with and without soluble thrombomodulin (ETP TM+ and ETP TM– respectively) (Chantarangkul et al. 2003). t-PA, D-dimer and C-reactive protein (CRP) were determined using commercially available ELISA assays (ELISA-Zymutest; HYPHEN BioMed, Neuville-Sur-Oise, France) (Bonzini et al. 2010).

2.5. Determination of 8-OH-dG content in mtDNA.

The content of 8-OHdG in mtDNA was measured on each sample with and without hOGG1 pre-treatment, using a quantitative real-time PCR to amplify different fragments of mtDNA as previously published (Lin et al. 2008). All experimental DNA samples were assayed in triplicates and the mean value of Ct was used in the statistical analyses.

2.6. miRNA profiling

RNA was converted to cDNA from Universal RT primers (First strand cDNA, Qiagen) using standard reverse transcription conditions. 8-Channel Liquid Handler (Microlab Starlet, Hamilton Robotics) was used to increase throughput and reduce error. To analyze miRNAs expression we used The miFinder RT² miRNA PCR Arrays (SA Bioscience) that measures the expression of the 88 most abundantly expressed among different tissues and best characterized micro RNA sequences by SYBR green chemistry genes (see Table 1S, Supplementary Material). Real-time PCR was performed using RT² qPCR MasterMix (Qiagen) and standard conditions. As there is no known control miRNA in MVs, several internal controls were examined. Primers to the internal controls, small nucleolar (sno)RNA SNORD48, SNORD47 and SNORD44 U38B as well as small nuclear RNU6–2 were included in the plates.

2.7. Statistical and network analysis

2.7.1. Associations between exposures and EVmiRNAs expression.—Linear regression models were used to evaluate the association between exposure to PM and metals and EVmiRNAs expression. To compare the magnitude of the associations between exposure to PM and metals and EVmiRNAs expression we calculated standardized regression coefficients. Adjustment for multiple testing was performed by Benjamini and Hochberg (1995) stratified sampling by EVmiRNAs. Correlations with a False Discovery Rate (FDR) P- value <0.1 was considered significant. Multivariate models included the following potential confounders: age, body mass index(BMI), current smoking habits, nonsteroidal anti-inflammatory agents/analgesics (NSAIDs) use, and percent monocytes.

2.7.2. Associations between EVmiRNAs expression and outcomes related to oxidative stress, coagulation and inflammation markers.—A linear mixed effect model was applied to evaluate the relationship of EVmiRNAs expression in circulating extracellular vesicles with coagulation and inflammatory markers, i.e. 8-OH-dG, t-PA antigen, D-dimer, ETP TM+, ETP TM–. The association of EVmiRNAs expression with coagulation and inflammation markers was evaluated regardless of whether the outcome measures were measured at sample 1 and sample 2.

$$Y_{ij} = \beta_0 + \beta_1(\text{miRNA expression})_j + \xi_{ij} + \epsilon_{ij}$$

where β_0 is the overall intercept; β_1 is the regression coefficient representing the miRNA expression ; $i=1,2,\dots,55$ represents the subject; $j=1,2$ represents the sample; ξ_{ij} is the random effect for sample and each subject and ϵ_{ij} is the residual error term. Likelihood ratio tests were used to test for the significance of β_1 .

The confounders were selected a priori and included general characteristics potentially associated with inflammatory processes: age, BMI, smoking, NSAID, and percent monocytes.

2.8. Bioinformatic analysis

A biological interpretation of EVmiRNAs expression data was done using Ingenuity Pathway Analysis (IPA) software as previously described (Bollati et al. 2015). IPA was queried for target mRNAs that were experimentally observed or predicted with a high confidence level and a Core Analysis was run using the target gene lists.

The top five networks, functions and molecules for ontologies that are related to inflammatory processes, immune functions and processes related to cardiovascular system development were considered.

2.9. Expression miRNAs in peripheral blood leukocytes and EVmiRNAs.

To evaluate if EVmiRNAs may be similar to miRNAs in leukocytes, the results previously obtained on miR-21, miR-146a and miR-222 expression in peripheral blood leukocytes (Wright et al. 2010) were compared with miR-21, miR-146a and miR-222 expression now measured in EVmiRNAs, to exclude results found that were an artifact due to leukocytes contamination. Pearson correlation coefficient was calculated to evaluate the correlation between miRNAs expression in the two biological compartments.

In described analysis, regression assumptions were checked to verify normality and the homogeneity of variance of the residuals. In order to satisfy the normality assumptions of linear regression models both miRNA expression and exposure were log-transformed.

All statistical analyses were performed in SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Characteristics of Study Participants

The characteristics of the 63 study subjects are summarized in Tables 1s and 2s. All study participants were males with mean age of 44 years (range: 27–55 years). All subjects lived around the steel plant factory. For both PM and metal levels, the participants showed wide ranges of exposures. The worker with the highest personal exposure level was at least 17-times more exposed than the one with the lowest exposure level (e.g., for PM10, the maximum level was 1220.2 $\mu\text{g}/\text{m}^3$ vs. minimum level of 73.7 $\mu\text{g}/\text{m}^3$). Additional details on the study group have been reported in Tables 1s and 2s (Tarantini et al. 2009; Cantone et al.

2011). Age, body mass index, smoking, NSAID, % monocytes use of anti-inflammatory medicaments were included in multivariate models with no observed differences.

3.2. Associations between individual exposure to PM and metal with expression of EVmiRNAs

The associations between individual long-term exposure to PM and PM-related metals with expression of EVmiRNAs were evaluated. Significant associations between 17 EVmiRNAs expression (mir-125a-5p-T, mir-126, mir-143, miR-181b, mir-194, mir-196b, miR-200c, mir-21, mir-25, miR-26b, miR-27b, miR-302a, miR-302b, mir-30a, miR-30d, miR-424, miR-9) and PM and metal exposure are reported in Table 1. Significant and non-significant associations are reported in Supplemental Information (Table 3S). Briefly, it has been found that all the significant associations were positive (Raw p-value<0.05 and FDR p-value<0.1) except for one negative between Barium and miR-30a. Mir-196b that resulted in being the most related to metal exposure (i.e., Antimony, Arsenic, Copper, Iron, Lead, Molybdenum, Nickel, Tin and Zinc).

3.3. Associations of significantly exposure-related EVmiRNAs expression with outcomes related to oxidative stress, coagulation and inflammation pathways.

When significantly exposure-related EVmiRNAs were correlated with individual outcomes linked to oxidative stress, coagulation and inflammation pathways, there were 3 EVmiRNAs significantly associated (i.e, miR-200c, miR-302b, miR-30d) as reported in Table 2. None of the considered outcomes were related to Mir-196b, which was the most associated with PM and metal exposure. Significant and non-significant associations are reported in Supplemental Information (Table 4S). In particular, when considering inflammatory markers, there originated a significant negative association between WBC and miR-200c ($\beta = -0.28$, 95%CI: -0.45 ; -0.11 , p-value<0.001), this miR-200c was highly related to Cadmium exposure in a covariate-adjusted positive association ($\beta = 0.30$, 95%CI: 0.13 ; 0.48 , p-value<0.001, see Table 1). When coagulation outcomes were considered, significant positive associations between ETP TM + and miR-302b were found ($\beta = 0.23$, 95%CI: 0.05 ; 0.42 , p-value=0.01), and also important outcomes were demonstrated between t-PA and miR-30d ($\beta = 0.24$, 95%CI: 0.06 ; 0.41 , p-value=0.01). MiR-302b was found to be highly related (see Table 1) to Cadmium ($\beta = 0.15$, 95%CI: 0.05 ; 0.26 , p-value<0.001), Lead ($\beta = 0.20$, 95%CI: 0.05 ; 0.36 , p-value<0.001) and Zinc exposure ($\beta = 0.23$, 95%CI: 0.08 ; 0.38 , p-value<0.001), while miR-30d to Aluminium exposure showed a covariate-adjusted association ($\beta = 0.20$, 95%CI: 0.04 ; 0.36 , p-value=0.01).

3.4. Prediction and analysis of miRNA targets

Three EVmiRNAs (miR-200c, miR-302b, miR-30d) that resulted to be associated with coagulation and inflammation markers were selected for downstream target prediction analysis with IPA.

To explore the functional role of the investigated EVmiRNAs and related targets we used MicroRNA Target Filter that provides experimentally validated as well as predicted microRNA-mRNA interactions from TargetScan, TarBase, miRecords and Ingenuity®

Knowledge Base. Only the miRNA-mRNA relationships that were experimentally observed and predicted with high confidence were considered.

The analysis identified 4067 mRNA that were experimentally observed or predicted high confidence level. More specifically, the study identified that miR-200c, miR-302b and miR-30d respectively regulated 1236, 1239 and 1592 targets.

IPA core analysis on miR-200c, miR-302b and miR-30d targets, recognized five primary networks with functions related to 1) connective tissue disorders, developmental disorder, hereditary disorder; 2) connective tissue disorders, developmental disorder, neurological disease; 3) cell morphology, cellular function and maintenance, hereditary disorder; 4) cellular development, embryonic development, organismal development; 5) hereditary disorder, neurological disease, psychological disorders. To test for cross talk among networks targeted by differentially-expressed miRNAs, we used the IPA algorithm for overlapping and merging networks. The analysis of networks involved in top molecular and cellular functions showed that out of the 25 networks identified as significant by the software, all of them are predicted to be involved in the cross-talk with the peripheral molecules bridging different networks. In particular, in Figure 1S, is shown the cross talk between miR-30d and PKC protein kinase C (PKC) a central member of the protein kinase C (PKC) family of serine/threonine kinases implicated in several physiological processes regulating the activation response of platelets, pathway in coagulation.

A Tox Lists analysis was performed with IPA to uncover pathways and molecular mechanisms that may be altered by toxicant exposure to PM and PM metal components. According to the p-value and the Overlap Score (%), the three EVmiRNAs (miR-200c, miR-302b and miR-30d) targets mapped to five toxicological pathways, belonging to cardiac hypertrophy, cardiac necrosis/cell death, p53 signaling, renal necrosis/cell death and hypoxia-inducible factor signaling (Table 3).

3.5. Expression of miRNAs in peripheral blood leukocytes and of EVmiRNAs

The study evaluated whether EVmiRNAs expression could have resulted from contamination of leukocytes. The expression of three microRNAs previously analyzed in peripheral blood leukocytes (Wright et al. 2010) were compared with the same three microRNAs measured in EVs. The expression of the two compartments were found not to be associated, therefore unlikely the results are to be considered an artifact (miR-21: $r = -0.07$, $p\text{-value} = 0.60$; miR-146a: $r = -0.01$, $p\text{-value} = 0.96$; miR-222: $r = -0.03$, $p\text{-value} = 0.85$).

4. Discussion

In the present study, based on a healthy population of workers employed in an electric-steel plant facility, the expression of 88 EVmiRNA isolated from plasma after occupational continuous exposure to PM and metallic component was evaluated. Levels of exposure within the steel plant were sensibly higher than the ones seen for general environment (around twenty times), reflecting the different activities and working position within the steel plant. In fact some areas were directly exposed to the fumes originated by the steel mills (i.e. Blast furnaces), others were cabins separated by the open environment. This is an

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advantage of the present study, because the large range of exposure allows us to better investigate differences in the study outcomes related to PM increases. Moreover, possible extra-professional exposures are unlikely important to determinate the global burden of PM exposure for the enrolled subjects. Epidemiological studies linked both short- and long-term exposure to PM in urban environments with increased morbidity and mortality from CVD (Schwartz and Dockery 1992; Katsouyanni et al. 2001). Our findings are in line with the fact that metals constitute the primary toxic component of airborne PM - especially for PM from industrial sources - and have been associated with cardiovascular effect in experimental and epidemiological studies (Dockery et al. 1993; Pope et al. 2004). It has been previously shown that increased expression of miR-128 in plasma EVs was related to short-term metal-rich PM exposure (after 3 days of workplace PM exposure (Bollati et al. 2015). Moreover, according to Ingenuity Path way Analysis, miR-128 is part of a pathway to the short-term effect of coronary artery disease, such as cardiac hypertrophy and heart failure pathways. In the present work, the hypothesis that long-term exposure to PM and metallic component influences expression of plasmatic EVmiRNAs has been explored, and that the elicited response is correlated, in turn, with long-term health effects of PM exposure, i.e., oxidative stress, inflammation and coagulation alterations. To the best of our knowledge, this is the first study that shows environmental long-term exposure is able to modify expression levels of EVmiRNAs, thus suggesting a new potential mechanism involved in air pollution toxicity.

In this study it has been demonstrated that airborne metal exposure is positively associated with changes in 17 EVmiRNA expression (mir-125a-5p-T, mir-126, mir-143, miR-181b, mir-194, mir-196b, miR-200c, mir-21, mir-25, miR-26b, miR-27b, miR-302a, miR-302b, mir-30a, miR-30d, miR-424, miR-9) and that mir-196b was the most often associated with metal exposure (i.e., Antimony, Arsenic, Copper, Iron, Lead, Molybdenum, Nickel, Tin, Zinc). Recently it has been found that miR-196b is involved in a post-transcriptional regulation mechanism of insulin biosynthesis (Panda et al. 2014). Our finding endorses the emerging evidence that long-term exposure to PM metallic component contributes to the risk for developing T2D (Liu et al. 2015). A reduced metabolic insulin sensitivity among healthy adults was in fact proposed as a novel adverse health effect of long-term exposures to PM (Brook et al. 2013b).

Furthermore, 3 (miR-302b, miR-200c, miR-30d) out of these 17 miR were also related to the changes in inflammatory markers and coagulation function. In particular an increased expression of miR-302b, related to long-term exposure to Cadmium, Lead and Zinc, is in turn associated with the rise in ETP (TM+). ETP is defined as the net amount of thrombin that is generated in plasma in vitro as a function of the pro- and anticoagulant factors and increased levels have been proposed as an index of hypercoagulability (Tripodi et al. 2007; Wielders et al. 1997). In addition, an increased expression of miR-200c is also associated to long-term exposure to Cadmium and that miR-200c is in turn inversely associated with white blood cell count. This result may be explained by apoptosis induced by cadmium in leukocytes (Chatterjee et al. 2008). As a matter of fact, it is known already a proapoptotic role for miR-200c (Magenta et al. 2011] both from studies in tumor and endothelial cells. On the other hand, miR-200c was found to be one of the four miRNAs able to regulate fibrinogen production (Fort et al. 2010], evoking a key role in coagulation pathways. Fine-tuning regulation of fibrinogen is of clinical interest because mild changes in fibrinogen

levels can influence risk of coronary heart disease and stroke. Indeed, epidemiologic studies have calculated that the hazard ratio for coronary heart disease and stroke is as high as 1.8 for every 1-g/L increase in plasma fibrinogen levels (Danesh et al. 2005).

An increase in miR-30d expression associated with Aluminum exposure was related with increased levels of t-PA antigen, consistently with previous results (Bollati et al. 2015). An increased level of t-PA antigen indicates a reduced fibrinolytic activity. In fact, the immunoassay used to measure t-PA antigen measures the circulating complexes between t-PA and the main fibrinolytic inhibitor PAI-1, reflecting a condition of systemic inflammation (Bonzini et al. 2010). Moreover, by IPA analysis, miR-30d was found to be a target that is part of protein kinase C (PKC) family pathways that are implicated in several coagulation physiological processes by regulating the activation response of platelets (Cohen et al. 2011; Strehl et al. 2007).

In the present study, assessment of a male working population with well-characterized exposure to PM (PM₁₀, PM₁ and PM metallic components) that allowed for contrasting subjects over a wide range of different exposure levels and different exposure types was done. Because of the limited number of study subjects, it is possible that the associations observed were due to chance. However, the occupational exposure and relatively controlled environment of a foundry provide a good setting for evaluating these mechanistic questions and reduce bias and chance findings. Moreover age, body mass index, smoking, NSAID, %monocytes use of anti-inflammatory medicaments were included in multivariate models with no observed differences. This study was based on subjects working in several work areas of the same factory but did not include a different population of subjects without a specific condition of exposure to PM. Limiting our investigation to individuals who have been working in the same work facility avoided potential concerns related to the selection of external referents who might have differed from the exposed population in terms of socioeconomic factors and other characteristics determining hiring into the plant. The choice of avoiding the use of personal sampler was done for safety reasons only, to avoid any possible interference of wearing additional devices with work activity and safety procedures. The differences in the individual levels of exposure within our study group turned out to be large, thus providing sufficient contrast for identifying exposure-related changes in miRNA expression (Wright et al. 2010). Another further limitation that needs to be acknowledged and addressed regarding the present study concerns the lack of feasibility of characterization of EVs origin. Whereas flow cytometer can be used to separate circulating EVs based on source tissues, this can be done only on fresh plasma. In our study, the blood samples were collected and first processed in the foundry, thus not allowing for tissue-specific separation. The second limitation concerns the number of miRNAs that were able to be examined. Detection of miRNAs by real-time PCR analysis can be considered one strong point of the present research. This approach was used, because real-time PCR has higher precision and sensitivity, compared to microarray technology, and this would make the study more efficient in detecting the effects of PM and its metal component. Real-time PCR is by many considered the 'gold standard' for gene expression analysis and commonly used for validation of microarray results (Mackay et al. 2002). Currently available microarrays have lower precision than real-time PCR in detecting miRNA expression and lower specificity in differentiating the expression of closely related miRNAs that differ in sequence by only one

base. Moreover, real-time PCR analysis appeared more efficient because of higher analytical sensitivity and specificity; reduction of the number of statistical comparisons, which translates in higher statistical power to detect potentially moderate effect sizes. The problem of multiple comparisons, which always arises when at the same time many associations are tested, is obviated through the control of the false discovery rate FDR using the Benjamini-Hochberg procedure that adjusts p-value and allows to protect themselves from the possibility of false positives. The use of this approach to control the proportion of “discoveries” (significant results) that are actually false positives with a cut-off for the adjusted p-value < 0.1 gives greater strength to the evidence that has emerged from the study. Another positive aspect of the study is that whether microRNAs expression in plasma EVs could have resulted from contamination from leukocytes was appraised. The comparison of expression of the three microRNAs previously analysed in peripheral blood leukocytes (Wright et al. 2010) with the same three microRNAs measured in EVs demonstrated that the expression of the two compartments was not associated, making the results unlikely to be an artefact.

Because available literature on EVmiRNAs carried in plasma is limited and there is not a well validated housekeeping to normalize miRNA expression data, 4 controls (SNORD48, SNORD47, SNORD44, and RNU6–2) were selected. They have been widely used to normalize miRNAs in different tissues and were included in each of the plates. By statistical methods, their variability of each housekeeping gene was evaluated through samples and, to reduce bias caused by using an arbitrary housekeeping as a normalization factor, the data was normalized with an average of the 4 controls.

The majority of the plasma EVs from normal subjects are derived from blood cells (Hunter et al. 2008). However, miRNAs and EVs in plasma may be frequently released upon tissue damage and have a specific function being delivered to their target cells (Hunter et al. 2008). The identification of miRNAs in plasma EVs of healthy subjects (Hunter et al. 2008) provides the basis for a new potential mechanism that may operate to produce cardiovascular effects and insulin resistance alteration, since EVs produced by lung or dendritic cells might be able to transfer a specific pattern of miRNAs to immune and endothelial cells.

Plasma is an easily obtainable biological medium that, if successful in identifying EVmiRNAs alterations, would be amenable to use in clinical settings and in exposed populations for potential future preventive and diagnostic applications. In addition, as miRNAs in EVs may potentially be a drug target, they might also open paths to develop future interventions to reverse at least some of the effects of air pollution.

These findings, throw light into the novel field of EVmiRNAs linked to PM exposure, CRD and T2D biology. Air particles continuous exposure, particularly the metallic component, is able to modify several EVmiRNAs and that three of these were also linked to inflammatory markers and coagulation functions. The involvement of miR-196b, the most often related to metal exposure, in insulin biosynthesis regulation, suggests to further investigate the role in long-term enriched-metals PM exposure to the risk for developing T2D also among foundry workers. The hypothesis that adverse cardiovascular and metabolic effects stemming from inhalation exposures in particular to PM metallic component may be mediated by

EVmiRNAs that target key factors in the inflammation, coagulation and glucose homeostasis pathways is strongly supported by this study. Additional studies specifically designed to investigate EVmiRNAs are required to elucidate EVs origin and miRNA profiling in a larger population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

PM	particulate air pollution
CRD	cardiorespiratory diseases
T2D	type 2 diabetes
EVs	Extracellular vesicles
EVmiRNAs	Extracellular vesicles-encapsulated microRNAs
IPA	Ingenuity Pathway Analysis
CRP	C-reactive protein
8-OH-dG	8-hydroxyguanine
ETP TM+	endogenous thrombin potential with soluble thrombomodulin
ETP TM–	endogenous thrombin potential without soluble thrombomodulin

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Table 1:

Significant Associations of PM mass and PM metal average level and average miRNA expression. Non-significant associations are reported in Supplemental Information, Table 1S.

Exposure	miRNA	Unadjusted			Adjusted ^a		
		β^b	95%	P-value	β^b	95%	P-value
Aluminium	mir-126	0,22	(0,06; 0,38)	0,01	0,22	(0,06; 0,38)	0,01
	mir-143	0,22	(0,03; 0,42)	0,03	0,22	(0,03; 0,42)	0,02
	mir-21	0,25	(0,09; 0,42)	0,00	0,27	(0,11; 0,43)	0,00
	miR-26b	0,19	(0,05; 0,33)	0,01	0,20	(0,06; 0,34)	0,01
	miR-27b	0,18	(0,05; 0,32)	0,01	0,19	(0,05; 0,32)	0,01
	miR-30d	0,19	(0,03; 0,34)	0,02	0,20	(0,04; 0,36)	0,01
	miR-424	0,05	(0,01; 0,1)	0,01	0,06	(0,02; 0,1)	0,00
Antimony	miR-181b	0,17	(0,04; 0,3)	0,01	0,17	(0,04; 0,3)	0,01
	mir-196b	0,25	(0,05; 0,45)	0,02	0,28	(0,06; 0,5)	0,01
	miR-9	0,21	(0; 0,41)	0,05	0,26	(0,03; 0,48)	0,03
Arsenic	mir-196b	0,24	(0,04; 0,44)	0,02	0,23	(0,02; 0,43)	0,03
	miR-9	0,24	(0,03; 0,44)	0,02	0,24	(0,03; 0,44)	0,03
Barium	mir-30a	-0,27	(-0,44; -0,09)	0,00	-0,29	(-0,47; -0,1)	0,00
Cadmium	mir-125a-5p-T	0,16	(0,04; 0,27)	0,01	0,16	(0,04; 0,28)	0,01
	mir-194	0,08	(-0,03; 0,2)	0,15	0,16	(0,01; 0,31)	0,03
	miR-200c	0,31	(0,14; 0,49)	0,00	0,30	(0,13; 0,48)	0,00
	miR-302a	0,15	(0,04; 0,25)	0,01	0,15	(0,05; 0,26)	0,01
	miR-302b	0,23	(0,08; 0,39)	0,00	0,22	(0,07; 0,38)	0,01
Coarse	mir-143	0,23	(0,03; 0,42)	0,02	0,23	(0,04; 0,42)	0,02
	mir-21	0,08	(-0,05; 0,22)	0,22	0,26	(0,11; 0,42)	0,00
	mir-25	0,15	(0,03; 0,27)	0,02	0,14	(0,02; 0,26)	0,02
Copper	mir-196b	0,27	(0,07; 0,47)	0,01	0,26	(0,06; 0,46)	0,01
Iron	mir-196b	0,30	(0,08; 0,51)	0,01	0,28	(0,06; 0,5)	0,01
	miR-9	0,24	(0,04; 0,45)	0,02	0,29	(0,06; 0,52)	0,01
Lead	miR-181b	0,20	(0,07; 0,32)	0,00	0,20	(0,07; 0,33)	0,00

Unadjusted					Adjusted ^a			
Exposure	miRNA	β^b	95%	P-value	β^b	95%	P-value	FDR
	mir-194	0.12	(0.01; 0.23)	0.03	0.17	(0.03; 0.32)	0.02	0.15
	mir-196b	0.27	(0.04; 0.49)	0.02	0.25	(0.02; 0.47)	0.03	0.07
	miR-302b	0.20	(0.05; 0.35)	0.01	0.20	(0.05; 0.36)	0.01	0.07
	miR-9	0.25	(0.05; 0.45)	0.02	0.31	(0.09; 0.54)	0.01	0.06
Manganese	miR-9	0.23	(0.03; 0.44)	0.03	0.24	(0.03; 0.45)	0.03	0.06
Molybdenum	mir-196b	0.25	(0.05; 0.45)	0.01	0.24	(0.04; 0.45)	0.02	0.06
	miR-9	0.24	(0.03; 0.44)	0.02	0.24	(0.03; 0.45)	0.03	0.06
Nickel	miR-181b	0.15	(0.02; 0.28)	0.03	0.14	(0.01; 0.27)	0.04	0.11
	mir-196b	0.24	(0.04; 0.44)	0.02	0.23	(0.03; 0.44)	0.03	0.07
PM ₁₀	mir-143	0.22	(0.03; 0.41)	0.02	0.23	(0.04; 0.42)	0.02	0.14
	mir-21	0.08	(−0.05; 0.22)	0.23	0.26	(0.1; 0.42)	0.00	0.01
	mir-25	0.15	(0.03; 0.27)	0.02	0.14	(0.02; 0.26)	0.02	0.15
Tin	mir-196b	0.25	(0.05; 0.45)	0.01	0.24	(0.04; 0.45)	0.02	0.06
	miR-9	0.23	(0.02; 0.43)	0.03	0.24	(0.03; 0.45)	0.03	0.06
Zinc	miR-181b	0.23	(0.11; 0.35)	0.00	0.23	(0.11; 0.36)	0.00	0.01
	mir-194	0.09	(−0.02; 0.2)	0.12	0.16	(0.02; 0.31)	0.03	0.15
	mir-196b	0.38	(0.16; 0.61)	0.00	0.38	(0.14; 0.61)	0.00	0.04
	miR-302b	0.23	(0.08; 0.38)	0.00	0.23	(0.08; 0.38)	0.00	0.05
	miR-9	0.29	(0.09; 0.49)	0.01	0.36	(0.14; 0.58)	0.00	0.03

^aMultivariable mixed models adjusted for age, body mass index, smoking, NSAID, % monocytes.

^bStandardized regression coefficients.

Table 2:

Significant associations of miRNA expression with blood coagulation and inflammation markers in a multivariable mixed models ^a. Non-significant associations are reported in Supplemental Information, Table 2S.

miRNA	Outcome	β_{std}^b	95%CI	p-value	FDR
miR-200c	WBCs (1000 mm ⁻³)	-0,28	(-0.45; -0.11)	0,00	0,01
miR-302b	ETP TM + (nM min)	0,23	(0.05; 0.42)	0,01	0,13
miR-30d	t-PA antigen (ng mL ⁻¹)	0,24	(0.06; 0.41)	0,01	0,08

t-PA antigen, tissue-type plasminogen activator; ETP, endogenous thrombin potential; ETP measured without exogenous triggers and with (TM+) thrombomodulin, WBCs (1000 mm⁻³) white blood cell.

^a Multivariable mixed models adjusted for age, body mass index, smoking, NSAID, %monocytes.

^b Standardized regression coefficient

Table 3:

Toxicological pathways for the three miR-200c, miR-302b and miR-30d targets identified by IPA Software Analysis.

Name	p-value	Pathway overlap (n/total)
Cardiac Hypertrophy	$1,03 \times 10^{-7}$	25,8 % (103/399)
Cardiac Necrosis/Cell Death	$7,01 \times 10^{-6}$	26,1 % (70/268)
p53 Signaling	$1,05 \times 10^{-5}$	33,3 % (33/99)
Renal Necrosis/Cell Death	$1,77 \times 10^{-5}$	22,8 % (113/496)
Hypoxia-Inducible Factor Signaling	$3,24 \times 10^{-5}$	35,7 % (25/70)