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Extracellular vesicles and their miRNA contents counterbalance the pro-inflammatory effect of air pollution during physiological pregnancy: A focus on Syncytin-1 positive vesicles

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ABSTRACT

The impact of exposure to respirable particulate matter (PM) during pregnancy is a growing concern, as several studies have associated increased risks of adverse pregnancy and birth outcomes, and impaired intrauterine growth with air pollution. The molecular mechanisms responsible for such effects are still under debate. Extracellular vesicles (EVs), which travel in body fluids and transfer microRNAs (miRNAs) between tissues (e.g., pulmonary environment and placenta), might play an important role in PM-induced risk. We sought to determine whether the levels of PM with aerodynamic diameters of ≤10 μm (PM_{10}) and ≤2.5 μm ($PM_{2.5}$) are associated with changes in plasmatic EV release and EV-miRNA content by investigating 518 women enrolled in the INSIDE study during the first trimester of pregnancy. In all models, we included both the 90-day averages of PM (long-term effects) and the differences between the daily estimate of PM and the 90-day average (short-term effects). Short-term PM_{10} and $PM_{2.5}$ were associated with increased concentrations of all seven EV types that we assayed (positive for human antigen leukocyte G (HLA-G), Syncytin-1 (Sync-1), CD14, CD105, CD62e, CD61, or CD25 determinants), while long-term PM_{10} showed a trend towards decreased EV concentrations. Increased Sync-1 + EV levels were associated with the plasmatic decrease of sVCAM-1, but not of sICAM-1, which are circulating biomarkers of endothelial dysfunction. Thirteen EV-miRNAs were downregulated in response to long-term PM_{10} and $PM_{2.5}$ variations, while seven were upregulated (p-value < 0.05, false discovery rate p-value (qFDR) < 0.1). Only one EV-miRNA (hsa-miR-221-3p) was downregulated after short-term variations. The identified PM-modulated EV-miRNAs exhibited putative roles in inflammation, gestational hypertension, and pre-eclampsia,

Abbreviations: ADM, adrenomedullin; AGT, angiotensinogen; AmpScore, amplification score; APLN, apelin; ARPA Lombardy, Regional Environmental Protection Agency Lombardy; BMI, body mass index; CI, confidence interval; CRH, corticotropin-Releasing Hormone; CT, cycle threshold; EDN1, endothelin 1; EDTA, ethylenediaminetetraacetic acid; EV, extracellular vesicles; EV-miRNA, EV miRNA content; FLT1, fms Related Receptor Tyrosine Kinase 1; HLA-G, human leukocyte antigen G; IL10, interleukin 10; IL6, Interleukin 6; IQR, interquartile range; LCI, lower confidence interval; LEP, leptin; miRNA, microRNAs; NTA, nanoparticle tracking analysis; PBS, phosphate-buffered saline; PGF, placental growth factor; PM, particulate matter; PM10, PM with aerodynamic diameters of ≤ 2.5 μm; PM2.5, PM with aerodynamic diameters of ≤ 10 μm; Q1, first quartile; Q3, third quartile; qFDR, False Discovery Rate; RQ, relative quantification; RT-qPCR, Real-time quantitative PCR; SD, standard deviation; SE, standard error; SERPIN1, serpin1; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, circulating vascular cell adhesion molecule-1; Sync-1, Syncitin-1; Treg, regulatory T lymphocytes; UCI, upper confidence interval; VEGFA, vascular endothelial growth factor A; Δ%, percentage change.

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as highlighted by miRNA target analysis. Our findings strongly support the hypothesis that EVs have an important role in modulating PM exposure effects during pregnancy, possibly through their miRNA cargo.

1. Background

The impact of air pollution on health is a growing concern that affects millions of people worldwide because of its association with a broad spectrum of acute and chronic diseases (World Health 2021). Several studies support the hypothesis that exposure to air pollution can increase the risks of adverse pregnancy outcomes, such as hypertensive disorders in pregnancy, as well as birth outcomes (Hannam et al. 2014; Lee et al. 2013; Olsson et al. 2013; Winckelmans et al. 2015) and impaired intrauterine growth (Bell et al. 2007; Brauer et al. 2008; Glinianaia et al. 2004; Maisonet et al. 2001). Significant associations have been reported for exposure to high levels of air pollution during both early (Bobak 2000; Jalaludin et al. 2007; Ritz et al. 2007) and late gestation (Leem et al. 2006; Olsson et al. 2013; Rich et al. 2009; Suh et al. 2009). While epidemiological results indicate that higher levels of particulate air pollution are associated with lower birth weight and consequently with increased neonatal morbidity and mortality (Barker and Thornburg 2013; Behrman 2007), the molecular mechanisms responsible for such effects are still under debate.

We recently investigated the effects of both short- and long-term PM exposure during pregnancy on different markers of inflammation and endothelial dysfunction, such as the soluble intercellular adhesion molecule-1 (sICAM-1) and the soluble vascular cell adhesion molecule-1 (sVCAM-1), whose endothelial surface expressions and consequent release into the circulation are increased by proinflammatory cytokine activation (Mozzoni et al. 2022). Indeed, we hypothesize that the inhalation of particulate matter (PM) might impact maternal systemic vascular function, with a potential effect on the developing fetus (Weldy et al. 2014). The highly vascularized placenta is the primary component of the feto-maternal interface, regulating oxygen tension, nutrient levels, certain immune functions, and intrauterine growth (Arck and Hecher 2013; Ward et al. 2012; Webster and Abela 2007). Moreover, PM inhalation induces an inflammatory response that might target the maternal cardiovascular system and endothelium (Lee et al. 2013; Mobasher et al. 2013), thus potentially causing endothelial dysfunction, as the endothelium acquires a pro-coagulative, pro-inflammatory, and pro-vasoconstrictive phenotype. This process is considered a key element in the pathogenesis of pregnancy-related complications, such as pre-eclampsia (Chambers et al. 2001; Steegers et al. 2010).

Because only a very small fraction of fine and ultrafine particles are thought to enter the bloodstream, a cross-talk between the PM-exposed pulmonary environment and peripheral organs (e.g. placenta) may be responsible for the observed peripheral effects of PM exposure (Brook et al. 2014). Extracellular vesicles (EVs) might be the ideal candidates to mediate the effects of PM exposure on pregnancy because they can be produced by the respiratory system stimulated by exposure (Kesimer et al. 2009; Prado et al. 2008), then translocate into the systemic circulation (Orozco and Lewis 2010) and interact with remote tissues, such as the placenta and maternal immune system. Moreover, EVs have a well-known role during pregnancy; syncytiotrophoblast-derived EVs are released in increasing amounts during pathological pregnancy (Goswami et al. 2006; Knight et al. 1998) and interact with immune cells (Adam et al. 2017; Chuong 2018). Remarkably, Syncytin-1 + EVs produced by the placenta can modulate maternal immunity, potentially targeting immune cells (Holder et al. 2012).

In this context, we investigated the effects of PM exposure on EV release during the first trimester in 518 pregnant women of the INSIDE population (Ferrari et al. 2020) and hypothesized a role of PM-induced EVs in the modulation of endothelial function.

2. Methods

2.1. Study population

The INSIDE study population and protocol have been previously described (Ferrari et al. 2020). The eligibility criteria included: being older than 18 years at enrolment; experiencing a physiological pregnancy; being resident in Lombardy at the time of recruitment; agreeing to sign an informed consent and donate a blood sample. Exclusion criteria included: a previous diagnosis of cancer and cardiovascular diseases or other chronic diseases, such as multiple sclerosis, epilepsy, and schizophrenia. All of the 518 pregnant women included in the study were recruited between June 2014 and July 2019 at the "Clinica Mangiagalli", Fondazione IRCCS Ca' Granda—Ospedale Maggiore Policlinico in Milan, Italy. All participants provided signed written informed consent. The study design, research aims, and measurements were approved by the Ethics Committee "Comitato Etico—Milano Area 2" of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, 20122 Milan, Italy (approval number #318), in agreement with principles of the Helsinki Declaration.

2.2. PM exposure assessment

Daily PM_{10} and $PM_{2.5}$ concentrations were derived from the Open Data Lombardy Region (https://www.dati.lombardia.it) database, which contains daily estimates of municipal aggregate values calculated by the Regional Environmental Protection Agency (ARPA Lombardy). The assessment of pollutant concentrations is based on the ARIA Regional Modelling (https://www.aria-net.it), a chemical-physical model of air quality that simulates the dispersion and chemical reactions of atmospheric pollutants. It integrates data from the monitoring stations of the ARPA Lombardy air quality network, meteorological data, emissions, concentrations at the beginning of the simulation period, and trends in adjacent areas; covering the entire Lombardy territory with a grid of 4 × 4 km cells; providing daily mean estimates available from the website at municipality resolution. Each study subject was attributed the PM estimate at her municipality of residence on the day of enrolment and back to 90 days before. Daily meteorological data (i.e., temperature and relative humidity) were obtained from ARPA Lombardy monitoring stations. The 24-hour mean for temperature was calculated in degrees Celsius.

2.3. EV analysis

Isolation, purification, and characterization of EVs were performed by following the minimal information for studies of extracellular vesicles (MISEV) 2018 guidelines (Thery et al. 2018) as detailed in Supplementary Table S1.

2.3.1. Blood collection and isolation of EVs from plasma

Fasting blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes between 9 and 10 a.m. and processed within 2 h from the phlebotomy. EDTA-blood was centrifuged at 1200*g* for 15 min at room temperature to obtain platelet-free plasma. Plasma was further centrifuged at 1000, 2000, and 3000*g* for 15 min at 4 °C, and pellets were discarded to remove apoptotic bodies and cell debris.

To prepare the EV pellet for nanoparticle tracking analysis (NTA) and flow cytometry, 1.5 mL of fresh plasma was transferred into an ultracentrifuge tube (Quick-Seal®- Round-Top, Polypropylene, 13.5 mL-Beckman Coulter, Inc) that was then filled with phosphate-buffered saline (PBS) and filtered with a 0.10 μm pore size membrane

(StericupRVP, 0.10 μm, polyethersulfone filter- Merck Millipore) to minimize the background contribution of interfering particles. Plasma was then ultra-centrifuged (Beckman Coulter Optima-MAX-XP) at 110,000*g* for 75 min at 4 °C, to obtain an extracellular vesicles-rich pellet and decanted. The pellet was resuspended in 500 μl of triple-filtered PBS (0.10 μm pore-size). The EV pellet was kept at −80 °C until use.

2.3.2. NTA of EVs

NTA was carried out with the Malvern NanoSight NS300 system (Malvern Panalytical ltd., Malvern, UK), used to visualize EVs by laser light scattering (Cantone et al., 2021). For each sample, five 30-*sec* records were registered. NTA output was then analyzed with integrated NTA software (Malvern Panalytical ltd.), providing high-resolution particle size distribution profiles and EV concentration measurements. NTA EV data were expressed as 10^6 per 1 mL of plasma.

2.3.3. Flow cytometry of EVs

To determine EV cellular origins, immunophenotyping was achieved with the MACSQuant Analyser flow cytometer (Miltenyi Biotec, Bergisch Gladbach, DE) following the manufacturer's protocol (https://tinyurl.com/yr9f8mt2). The Fluoresbrite Carboxylate Size Range Kit I (0.2, 0.5, 0.75, and 1 μm) was used to set the calibration gate on the MACSQuant Analyser system. To evaluate the integrity and to highlight EV subsets, 60-μL sample aliquots were stained with 0.02 μM 5 (6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) at 37 °C for 20 min in the dark. CFSE is a vital non-fluorescent dye that enters EVs, where intracellular esterase enzymes remove the acetate group and convert the molecule into the fluorescent ester form. To characterize and count EVs, the following panel of antibodies were used: allophycocyanin- (APC-) conjugated primary monoclonal human anti- human antigen leukocyte antigen G (HLA-G) antibody (Clone MEM-G/9; dilution 1:10), and primary monoclonal IgG1 anti-syncytin-1 (Sync-1) antibody (Clone 4F10; dilution 1:60), detected with secondary APC-anti-IgG1 secondary antibody (dilution 1:10) to select EVs from trophoblasts; monoclonal APC-anti-CD14 (Clone TÜK4; dilution 1:10) to identify EVs from macrophages and/or monocytes; monoclonal APC-anti-CD105 (clone: 43A4E1; dilution 1:10) and monoclonal APC-anti-CD62e (clone REA280; dilution 1:10) to select EVs from endothelial and activated endothelial cells; monoclonal APC-anti-CD61 (clone: Y2/51; dilution 1:10) to select EVs from platelets; APC-anti-CD25 (clone: 3G10; dilution 1:10) to select EVs from regulatory T [T(reg)] lymphocytes. All antibodies were purchased from Miltenyi Biotec. Before use, each antibody was centrifuged at 17,000*g* for 30 min at 4 °C to eliminate aggregates. A stained PBS blank sample was used to detect auto-fluorescence of the antibody. Quantitative multiparameter analysis of flow cytometry data (expressed as 10^3 for 1 mL of plasma) was conducted using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Antibody gating strategies were previously described (Ferrari et al. 2019; Rota et al. 2020).

2.3.4. EV-miRNA analysis

miRNAs were isolated from frozen EV pellets by using the miRNeasy Kit and RNeasy CleanUp Kit (Qiagen, Hilden, DE). Briefly, 700 μl of QIAzol Lysis Reagent was added to each ultracentrifuge tube to lyse membrane particles. miRNA extraction was performed according to the manufacturer's protocol. The final purified miRNA-enriched RNA was eluted in 20 μl of RNAase-free water and stored at −80 °C until further use. miRNAs were prepared by standard reverse transcription (RT) and preamplification procedures, followed by real-time quantitative RT-qPCR analysis with the QuantStudio™ 12 K Flex OpenArray® Platform (QS12KFlex; Thermo Fisher Scientific, Waltham MA, USA), as previously described (Pergoli et al. 2017). Gene Expression Suite Software (Thermo Fisher Scientific) was used to process miRNA expression data from the miRNA panel.

2.4. Measure of markers of endothelial dysfunction

After plasma separation from whole blood, as described above, circulating concentrations of endothelial dysfunction biomarkers (sICAM-1 and sVCAM-1) were determined by enzyme-linked immunoassay (ProteinSimple, San Jose, CA, USA) using the Ella Automated Immunoassay System inside a patented glass nano-reactor based on a microfluidic platform (ProteinSimple, San Jose, CA, USA).

2.5. Statistical analysis

Descriptive statistics were performed on all variables. Continuous variables were expressed as the mean ± standard deviation (SD), median with first, and third quartile (Q1–Q3). Categorical data were reported as frequencies with percentages.

To evaluate the long-term effect of air pollution (i.e. basal level of maternal exposure experienced from conception to blood draw at the end of the first trimester of gestation) we calculated the 90-days PM_{10} and $PM_{2.5}$ averages, as the average of the exposure lags from Day0 (day of blood draw) to Day −90. This value of exposure was here defined as "long-term exposure".

To evaluate the short-term effects of PM_{10} and $PM_{2.5}$, we computed the difference between the PM levels experienced the day before the blood draw (Pergoli et al. 2017) and the long-term exposure. This exposure was defined as "short-term exposure".

We examined the association between PM exposure and EV subtype concentrations by applying multivariable linear regression models. In all models, we included both the long-term and the short-term exposures to take into account both the basal level of exposure and the deviation in air pollution levels from the 90-day average. EV counts and characterizations were naturally log-transformed to achieve approximately normal distributions of residuals. We derived the percentage of change (Δ%) and relative confidence interval in the EV types related to each PM exposure that was calculated using the formula: $\Delta\% = [(\exp(\beta-1))*100]$. Estimates were provided as β with standard error (SE) and with a Δ% in mean PM exposure for a 10 $\mu g/m^3$ increment.

The same statistical model was also adopted to investigate the association between the different EV types and the levels of the endothelial dysfunction sICAM-1 or sVCAM-1 markers. The two outcomes were log-transformed, and estimates were provided as Δ% for 1 interquartile range increment in each EV type. We accounted for *a priori* selected covariates that included known determinants of each outcome EV concentration or sICAM-1/sVCAM-1 markers and variables with a potential link with PM and endothelial dysfunction parameters such as age, gestational age at recruitment, body mass index (BMI), smoking habits (never smoked, stopped during pregnancy, smoker), temperature, relative humidity, and batch. This final set of covariates was selected after the usual preliminary inspection of the fit of each variable in turn in the model. EV concentration parameters were natural log-transformed, and each model was tested for linearity and normality of residuals. Best model selection was based on the minimization of the Akaike Information Criterion and maximization of the explained variance of the model.

A moderated mediation analysis was run using the PROCESS macro for SAS (Model 7) (Hayes and Rockwood 2017). Short-term exposure was put as a moderator of the relationship between long-term exposure and Sync-1 + EVs, which was put as a mediator of the relationship between long-term exposure and sVCAM-1. sVCAM-1 was the dependent variable. The analyses assessed: 1) the effects of long-term exposure on sVCAM-1 (both directly and indirectly, through Sync-1 + EVs; 2) the effect of long-term exposure on Sync-1 + EVs (as moderated by short-term exposure); 3) the effect of Sync-1 + EVs on sVCAM-1. Each model was also adjusted for age, gestational age at recruitment, BMI, smoking habits, temperature, relative humidity, and batch. The analysis combines mediation and moderation to estimate the conditional indirect effect of long-term exposure on sVCAM-1 through Sync-1 + EVs as moderated by short-term exposure. The indirect effect was calculated at

−1SD, the mean, and + 1SD of the moderating variable. The statistical significance of direct and indirect effects was evaluated by means of 10,000 bootstrap samples to create 95 % bias-corrected confidence intervals. An index of moderated mediation was used to test the significance of the moderated mediation, i.e., the magnitude of the difference between the indirect effects across levels of short-term PM. Significant effects are supported by the absence of zero within the confidence intervals.

For miRNA analysis, miRNAs with the cycle threshold (CT) value > 27 or amplification score (AmpScore) < 1.24, or missing were considered unamplified. miRNAs that were not amplified in all subjects were excluded, resulting in 274 miRNAs included in the analysis. The global mean was selected as the best normalization method and miRNA expression was determined using the relative quantification "$2^{-\Delta Ct}$ method", calculating the normalization factor as the geometric mean of the relative quantification (RQs) of all expressed miRNA per sample.

We used linear regression models to verify the association between PM_{10} and $PM_{2.5}$ exposure and miRNA expression. miRNA expression values were log2-transformed to achieve a normal distribution. Multivariable regression including both long- and short-term effects of PM was adjusted for age, gestational age at recruitment, BMI, smoking habits, temperature, and relative humidity. Due to the high number of comparisons, we applied a multiple comparison correction based on the False Discovery Rate (FDR) control. A threshold of 0.10 was applied to the FDR P-value (qFDR) significance level to identify the set of top miRNAs. All analyses were performed with SAS software version 9.4.

2.6. Identification of miRNA targets and bioinformatics analyses

To investigate the possible biological effects induced by each miRNA associated with PM exposure, miRNA target prediction was performed using the miRNAtap R package (Pajak, 2021). We considered *bonafide* miRNA-target interactions only those predicted by at least 2 algorithms among DIANA (Maragkakis et al. 2011), Miranda (Enright et al. 2003), PicTar (Lall et al. 2006), TargetScan (Friedman et al. 2009), and miRDB (Wong and Wang 2015) In addition, using the rank list we obtained from our target analysis, we performed an enrichment analysis to check the biological process and the Kolomonogorov Smirnov (K-S) test to test the significance (data not shown). Then, we used the DisGeNET database (v 7.0) to find genes related to 'Inflammation', 'pregnancy-associated hypertension', and 'pre-eclampsia' (Pinero et al. 2020). All the bioinformatic analyses were performed using R software (v 4.0.4).

Table 1
Description of the study population.

Characteristic	Total population (n = 518)
Age, year, mean ± SD (min; max)	33.8 ± 4.3 (19; 52)
Gestational age at sampling, weeks, mean ± SD (min; max)	11.9 ± 0.7 (9.7; 15.6)
BMI, Kg/m^2, mean ± SD (min; max)	22.4 ± 3.7 (15.6; 42.2)
Categorical BMI	
Underweight (BMI < 18.5)	50 (9.8 %)
Lean (18.5 ≤ BMI < 25)	358 (69.8 %)
Overweight (BMI ≥ 25)	105 (20.5 %)
Smoking habits, n (%)	
Never smoked	435 (84.0 %)
Stopped during pregnancy	55 (10.6 %)
Smoker	25 (4.8 %)
Parity	
Nulliparity	315 (60.8 %)
Multiparity	201 (38.8 %)

3. Results

3.1. Description of the study population

Table 1 summarizes the demographic characteristics of the 518 women enrolled in the INSIDE study. The mean maternal age was 33.8 years (SD = 4.3 years) and the mean body mass index (BMI) was 22.4 (SD = 3.7). The majority of women never smoked (84 %), while 10.6 % stopped smoking at the beginning of pregnancy. Pregnant women were enrolled during the first trimester with a mean gestational age at the sampling of 11.9 weeks (SD = 0.7), and most of them were primiparous (60.8 %). The estimated levels of long-term and short-term exposures to PM_{10}, $PM_{2.5}$, and other variables such as temperature and relative humidity at the time of recruitment, are described in Table 2. Short and long-term exposure to PM_{10} were not correlated (rho = -0.06, p-value = 0.15), while a weak correlation between short and long-term exposure to $PM_{2.5}$ was observed (rho = -0.14, p-value = 0.01). Both PM_{10} and $PM_{2.5}$ concentrations were higher than the annual limits set at 15 μg/m^3 and 5 μg/m^3, respectively (World Health 2021).

3.2. EV characterization and association with PM exposure

To evaluate the possible combined effect of long-term and short-term exposures to PM_{10} and $PM_{2.5}$ on healthy pregnancies, we first evaluated the total number of EVs present in maternal blood at the time of recruitment. As determined by NTA, the mean plasma EV diameter was 228.4 nm, with a mode of 172.2 nm. Total EV count descriptive statistics are reported in Table 3. Short-term exposures to elevated PM_{10}, but not $PM_{2.5}$, levels, were associated with decreased total EV concentrations. Indeed, a 10 μg/m^3 increase in PM_{10} predicted a −2.6 % (95 % CI: −5.0, −0.1, p = 0.043) decrease in total EV count. Long-term PM_{10} exposure showed a similar tendency (Δ%= −5.8 %, p-value = 0.066), although not significant (Supplementary Tables S2 and S3).

In addition to measuring total EV concentrations, we characterized seven EV types (Table 3): CD105+, CD62e+, CD14+, CD61+, CD25+, HLA-G+, and Sync-1 + EVs. The correlation coefficients obtained among the different EV types are reported in Supplementary Table S4. Fig. 1 and supplementary Tables S2 and S3 report the associations between long-term and short-term exposures to 10 μg/m^3 of PM_{10} and $PM_{2.5}$ and these seven EV types. The deviation in air pollution levels from the 90-day average (i.e., short-term PM_{10} and $PM_{2.5}$ exposures) were related to increased concentrations of all the seven EV types investigated, with significant associations for CD105+ (PM_{10}: Δ%= 5.1

Table 2
Description of exposure variables.

Exposure	Mean	Std	Median	First quartile	Third quartile
PM_{10} (μg/m^3)					
Long-Term[a]	35.4	11.3	35.0	25.0	45.0
Day −1	37.9	21.1	33.0	22.2	47.7
Short-term[b]	1.6	18.8	−0.8	−10.7	10.6
$PM_{2.5}$ (μg/m^3)					
Long-Term[a]	27.9	8.3	29.0	20.0	35
Day −1	29.2	13.5	27.8	18.4	36.9
Short-term[b]	0	12.6	−1.4	−9.2	6.9
Temperature (°C)[c]	14.3	7.5	13.5	8.1	19.1
Relative Humidity (%)[c]	65.4	16.2	65.0	52.9	76.7

[a] Long-term exposure is defined as the average exposure experienced by the mother, in the 90 days before blood draw (i.e. approximately from the conception).
[b] Short-term exposure is defined as the difference between the day before the blood draw and long-term exposure.
[c] Measured on the day of the blood draw.

Table 3
Description of measured EVs.

EV cellular origin*	Mean	Std	Median	First quartile	Third quartile	IQR
Sum EVs	2466.3	1535.3	2025.4	1374.4	3198.5	1824.1
CD105 + EVs (endothelium)	12	8.5	10.3	6.6	14.5	7.8
CD62e + EVs (endothelium)	16.1	10.1	14.5	8.9	20.7	11.8
CD14 + EVs (macrophages/monocytes)	35.4	45.9	23	14.1	38.6	24.4
CD61 + EVs (platelets)	115.8	126.1	84.9	53.1	137.2	84.0
CD25 + EVs (T(reg) lymphocytes)	8.9	5.8	7.6	4.9	11.4	6.6
HLA-G + EVs (trophoblasts)	19.9	13.4	17	11.4	24	12.6
Sync-1 + EVs (trophoblasts)	30.6	49.5	13.3	7.7	25.3	17.5

IQR: Interquartile range. EVs are measured on the day of the blood draw. *Sum of EVs obtained by NTA analysis is expressed as EV particles x10^6/mL; EV subtypes obtained by flow cytometry are expressed as EV particles x10^3/mL.

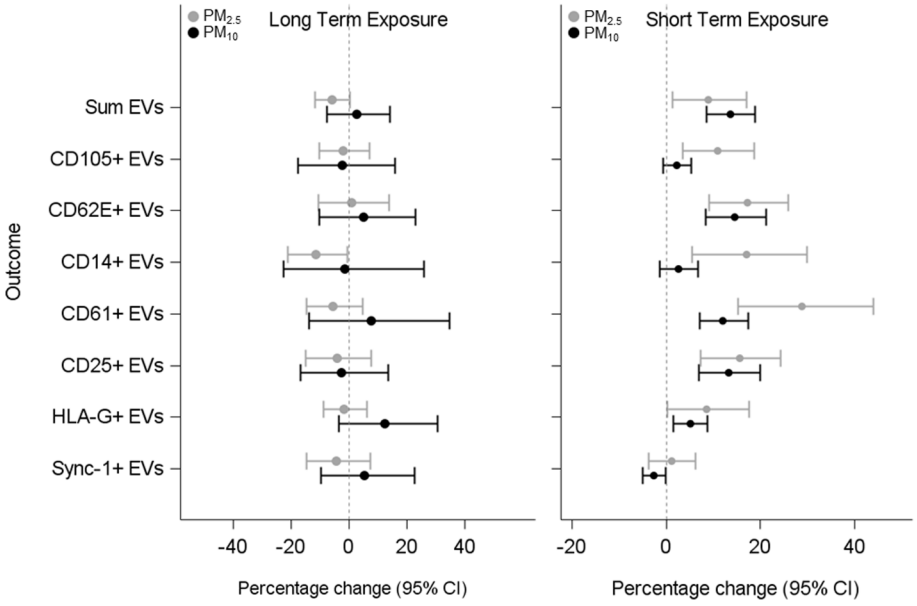

Fig. 1. Associations between long-term and short-term exposures to 10 μg/m^3 of PM_{10} and $PM_{2.5}$ and EV types. Models were adjusted for age, BMI, smoking habits, temperature, humidity, gestational age at recruitment, and batch. All outcome variables were log-transformed. Long-term exposure is defined as the exposure experienced by the mother in the 90 days before the blood draw (i.e., approximately from conception). Short-term exposure is defined as the difference between the day before the blood draw and long-term exposure.

%, p = 0.004; $PM_{2.5}$: Δ%= 8.6 %, p = 0.044), CD62e+ (PM_{10}: Δ%= 13.2 %, <0.001; $PM_{2.5}$: Δ%= 15.6 %, p < 0.001), CD14+ (PM_{10}: Δ%= 12.1 %, p < 0.001; $PM_{2.5}$: Δ%= 28.9 %, p < 0.001), CD25+ (PM_{10}: Δ%= 14.6 %, p < 0.001; $PM_{2.5}$: Δ%= 17.2 %, p < 0.001), and Sync-1 + EVs (PM_{10}: Δ%= 13.6 %, p < 0.001; $PM_{2.5}$: Δ%= 8.9 %, p = 0.020). In addition, a 10 μg/m^3 increase in $PM_{2.5}$ predicted a 17.1 % increase in CD61 + EVs (p = 0.003) and a 10.9 % increase in HLA-G + EVs (p = 0.003). In contrast, long-term exposure to PM_{10} generally showed a

Table 4
Association of EV cellular origin and sICAM-1 and sVCAM-1.

Outcomes	EV cellular origin	IQR[a,b]	Δ% x 1 IQR	LCI	UCI	*p-value*
sICAM-1	Sum EVs	1824.1	−5.04	−0.01	−0.02	**0.029**
	CD105 + EVs (endothelium)	7.8	1.68	0.25	3.77	0.149
	CD62e + EVs (endothelium)	11.8	−2.68	−0.79	−29.08	0.436
	CD14 + EVs (macrophages/monocytes)	24.4	0.79	0.03	1.02	0.364
	CD61 + EVs (platelets)	84.0	0.62	0.005	0.17	0.353
	CD25 + EVs (T(reg) lymphocytes)	6.6	2.51	1.45	109.56	0.512
	HLA-G + EVs (trophoblasts)	12.6	0.71	0.07	4.18	0.577
	Sync-1 + EVs (trophoblasts)	17.5	0.20	0.005	0.32	0.654
sVCAM-1	Sum EVs	1824.1	3.66	0.00	0.06	0.142
	CD105 + EVs (endothelium)	7.8	2.09	0.30	2.05	0.067
	CD62e + EVs (endothelium)	11.8	2.56	0.55	19.19	0.319
	CD14 + EVs (macrophages/monocytes)	24.4	−0.03	−0.001	−0.09	0.975
	CD61 + EVs (platelets)	84.0	0.80	−0.60	2.20	0.247
	CD25 + EVs (T(reg) lymphocytes)	6.6	5.38	2.24	13.40	0.056
	HLA-G + EVs (trophoblasts)	12.6	0.35	0.03	2.68	0.778
	Sync-1 + EVs (trophoblasts)	17.5	−1.44	−0.03	0.00	**0.001**

Models were adjusted for short-term PM_{10} and long-term PM_{10} exposures, age, BMI, smoking habits, temperature, relative humidity, gestational age at accrual, and batch. sICAM-1 and sVCAM-1 were log-transformed. [a]Sum of EVs obtained by NTA analysis are expressed EV particles x10^6/mL; [b]EV subtypes obtained by flow cytometry are expressed as EV particles x10^3/mL. Percentage of change (Δ%) and the relative confidence interval was calculated using the formula Δ%= [(exp(β-1)) *100] and reported for increasing 1 IQR for each EV type. LCI-UCI: lower- and upper- confidence interval; IQR: interquartile range.

trend toward lower EV concentrations, that was significant only for CD14 + EVs (Δ%= −11.3 %, p = 0.043).

3.3. EV types and association with sICAM-1/sVCAM-1 markers

As a further step, we investigated the association between the different EV types and levels of the plasmatic endothelial dysfunction sICAM-1 or sVCAM-1 markers (Table 4). The mean sICAM-1 and sVCAM-1 concentrations were 340 pg/ml and 787 pg/ml, respectively, and have been previously described in the same population (Mozzoni et al. 2022).

We observed that the total number of EVs was associated with a decrease in sICAM-1 (Δ%= −5.04 %, p = 0.029). Interestingly, increased levels of Sync-1 + EVs were associated with decreased sVCAM-1 (Δ%= −1.44 %, p = 0.001), but not sICAM-1. Moreover, long-term PM_{10} exposure was associated with sVCAM-1 levels (β = 0.058, p < 0.001; Table 5).

3.4. Moderated mediation analysis

We performed a moderated mediation analysis testing the indirect effect of long-term PM_{10} exposure on sVCAM-1 through Sync-1 + EV concentration, conditional upon short-term PM exposure (Fig. 2). Sync-1 + EV concentration was associated with an interaction effect of short- and long-term PM_{10} exposure (β = 0.064, p = 0.028), as reported in Table 5. Based on this assumption, we hypothesized that long-term PM_{10} exposure may lead to an increased concentration of Sync-1 + EV and a decreased sVCAM-1 level, in a manner moderated by short-term PM_{10}. The results reported in the Table 6 indicate that long-term PM_{10} is linked to an increased concentration of Sync-1 + EV when short-term PM_{10} is low (β[mean-SD] = 0.0063, 95 % bootstrapped confidence interval: 0.0001, 0.0182). The overall moderated mediation model was supported with the index of moderated mediation = -0.0022 (95 % CI = -0.0058; −0.0003) suggesting a potentially stronger mediational role of Sync-1 + EV on sVCAM-1 when short-term PM_{10} exposure is low.

3.5. Association between ambient PM levels and EV-miRNAs

OpenArray technology was applied to screen for EV-miRNA expression. Considering only miRNAs expressed in at least one subject, we examined 274 miRNAs. We evaluated the association between the expression of these miRNAs and the long-term/short-term exposure to PM_{10} and $PM_{2.5}$. Volcano plots describing these associations is reported in Fig. 3. In particular, we identified 20 miRNAs that were altered in response to long-term exposure to both PM_{10} and $PM_{2.5}$ (Fig. 4): 13 EV-miRNAs were downregulated, while 7 were upregulated (p-value < 0.05 and qFDR < 0.10). As we considered short-term exposure, only hsa-miR-221-3p showed a qFDR < 0.1. The presented results were normalized by the global mean miRNA expression. As a sensitivity analysis, we applied additional methods for normalization (four more stable miRNAs, and U6 small nuclear RNA). This step did not significantly affect our findings. For each miRNA, we considered genes predicted by at least two of the four evaluated algorithms to be *bona fide* target genes. The number of predicted target genes is reported in Supplementary Table S5. To elucidate the mechanisms through which the identified PM-modulated EV-miRNAs could impact pregnancy, genes from the miRNA-target analysis were compared to those found in association with "inflammation", "pregnancy-associated hypertension" and "pre-eclampsia" in the DisGeNET database. For the genes targeted by these miRNAs, we drew a Venn diagram showing those associated at least with one of the 3 conditions: inflammation, gestational hypertension, and pre-eclampsia (Fig. 5). Twelve genes were common to all 3 conditions: *ADM* (Adrenomedullin), *AGT* (Angiotensinogen), *APLN* (Apelin), *CRH* (Corticotropin-Releasing Hormone), *EDN1* (Endothelin 1), *FLT1* (Fms Related Receptor Tyrosine Kinase 1), *IL10* (Interleukin 10), *IL6* (Interleukin 6), *LEP* (Leptin), *PGF* (Placental Growth Factor), *SERPIN1* (Serpin1), and *VEGFA* (Vascular Endothelial Growth Factor A).

Table 5
Moderated mediation analysis.

	Path	β	SE	LCI	UCI	*p-value*
Outcome variabile: Sync-1 + EVs (trophoblasts)						
Long-term PM_{10}	a_1	−0.063	0.078	−0.220	0.092	0.424
Short-term PM_{10}	a_2	−0.152	0.129	−0.406	0.101	0.237
Long-term × short-term PM_{10}	a_3	0.064	0.029	0.007	0.121	0.028
Outcome variable: sVCAM-1						
Sync-1 + EVs	*b*	−0.035	0.013	−0.060	−0.010	0.006
Long-term PM_{10}	Direct effect (c')	0.058	0.017	0.025	0.091	<0.001

All models were also adjusted for age, BMI, smoking habits, temperature, relative humidity, gestational age at sampling, and batch.

4. Discussion

We observed a positive association between short-term exposures to $PM_{2.5}$ and PM_{10} and the number of EVs (Sync + EVs derived from trophoblasts, CD14 + EVs from macrophages, and/or monocytes, CD105 + and CD62E EVs from the endothelium, and CD25 + EVs from T(reg) lymphocytes). In contrast, long-term exposure was associated with a tendency towards EV reduction, also confirmed by a negative association with the total EV count. Of particular interest are the relationships among long-term exposure, short-term exposure, Sync + EVs, and sVCAM-1. In fact, long-term exposure to PM_{10} was linked to an increased concentration of Sync + EVs, which was in turn associated with a decrease in sVCAM-1, only if short-term exposure to PM_{10} was low. Finally, our analysis of EV miRNA content indicated that PM-modulated EV miRNAs might promote the pathogenesis of inflammation, pre-eclampsia, and gestational hypertension.

The general hypothesis of the present research was that PM exposure, chosen as a paradigmatic environmental stressor, might impact the EV signaling network, thereby altering maternal health, and eventually influencing newborn development. Pregnancy is characterized by chronic low-grade inflammation and by the need to strictly regulate acute inflammation (Palomba et al. 2014). Thus, the perturbation of this equilibrium by environmental triggers might be counterbalanced by physiological control mechanisms initiated during pregnancy.

The current literature describes a positive association between PM exposure and EV release in healthy non-pregnant subjects that might promote inflammation (Bonzini et al. 2017; Kong et al. 2020; Pergoli et al. 2017; Rota et al. 2020). We observed a similar finding of acute increases of seven EV subtypes following short-term exposures to PM. In particular, the proinflammatory role exerted by CD14 + EVs and endothelium-derived CD105 + and CD62E + EVs has been described previously (Benedikter et al. 2018; Pergoli et al. 2017; Rota et al. 2020). In addition, high levels of plasmatic EVs are generally measured after particulate pollution exposure since both PM-induced inflammation and oxidative stress increase the number of circulating vesicles (Emmerechts et al. 2012; Neven et al. 2017; Pope et al. 2016).

On the other hand, we also observed an increase in placenta-derived Sync-1 + EVs and T(reg) cell-derived CD25 + EVs. Sync-1 plays several recognized physiological roles during pregnancy (Chuong 2018). The "domestication" of the Syncytin-1 gene, which originates from a human endogenous retroviral virus (HERV) gene (*HERVW-1*) and retains lytic properties, is necessary for the development of the syncytiotrophoblast, which separates maternal and fetal bloodstreams in the placenta

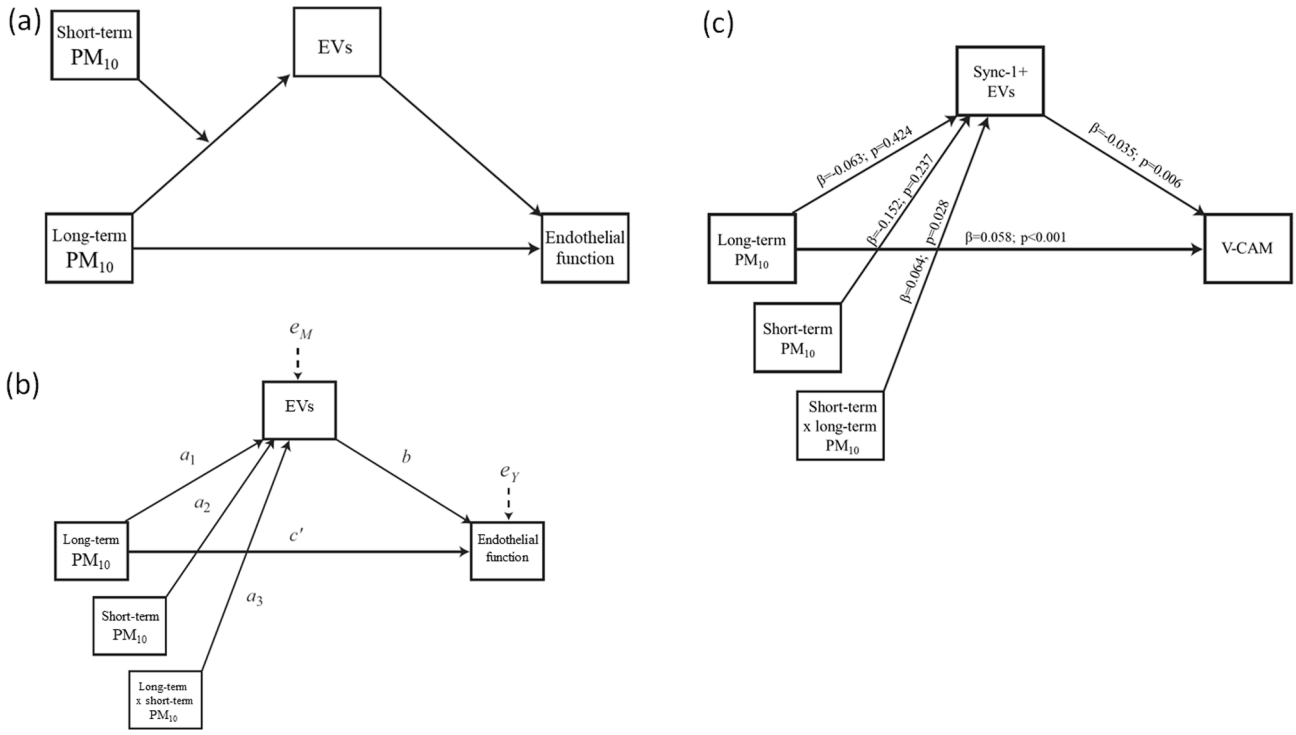

Fig. 2. Conceptual diagrams and diagram of the results from moderated mediation analysis. (a) Computational diagram; (b) statistical diagram; (c) diagram of the results.

Table 6
Moderator analysis: conditional indirect effect of long-term PM_{10} exposure on Sync-1 + EVs at selected values of short-term PM_{10} exposure.

Short-term PM_{10}	**Effect of PM long-term on** Sync-1 + EVs	**SE**	**LCI**	**UCI**
Low (mean - SD)	0.0063	0.0047	0.0001	0.0182
Medium (mean)	0.0021	0.0028	−0.0018	0.0095
High (mean + SD)	−0.0021	0.0029	−0.0091	0.0022
Index of Moderated Mediation	−0.0022	0.0014	−0.0058	−0.0003

95% bias-corrected bootstrapped confidence interval.

(Sugimoto and Schust 2009). Moreover, Sync-1 + EVs are produced by the placenta to suppress maternal immunity, permitting fetal development. For instance, Sync-1 suppresses the expressions of tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which are pro-inflammatory regulators linked to early pregnancy loss and pre-eclampsia (Nakahara et al. 2020; Southcombe et al. 2011). Although the details of this process are still unknown, the protective and immune-modulatory role of Sync-1 seems evident. As a possible counterpart to this observation, we also observed that short-term PM exposure is associated with an increased concentration of CD25 + EVs. CD25 + EVs are produced by T(reg) lymphocytes, a small population of cells that inhibit maladaptive immune responses (Shevach 2018). These vesicles can suppress effector T cell proliferation in a dose-dependent manner (Tung et al. 2020). T(reg) cells are increased in both the human decidua and circulation during pregnancy (Kahn and Baltimore 2010; Salvany-Celades et al. 2019; Wienke et al. 2020), thus reducing the risks of spontaneous abortion, pre-eclampsia, and spontaneous preterm birth (Hsu et al. 2012; Schjenken et al. 2020; Woidacki et al. 2015).

NTA analysis disclosed a surprising reduction of the total EV concentration in response to short-term PM exposures. This finding contrasts with the increase of circulating EVs after acute PM exposure in non-pregnant individuals described in previous reports, and the increased concentrations of the seven particular EV types assayed in our study. However, this apparent contradiction might find an explanation that focuses on the critical role of EVs in the modulation of inflammation, especially during pregnancy which is characterized by an extremely fine balance between pro-inflammatory and anti-inflammatory responses (Condrat et al. 2021). Chronic systemic inflammation during pregnancy has been linked to various adverse outcomes, for both the fetus and the pregnant woman, thus the effects of pro-inflammatory environmental stimuli have to be kept under control (Isaevska et al. 2021; Nair and Salomon 2020; Parisi et al. 2021). Hence, we speculate that if a non-pregnant individual can tolerate the inflammation produced by EV release after short-term PM exposure, the adaptive value of maintaining a tolerogenic condition during a physiological pregnancy might have a role in mitigating this phenomenon. Moreover, we hypothesize that our measurements of total EV concentrations included EV types that were not among the seven types characterized in this study, and which might have been reduced after short-term PM exposures. Interestingly, no effects on EV release were observed after long-term PM exposure. Thus, placenta-derived EVs might mediate the effects of long-term PM exposure on proinflammatory factors, such as sICAM-1 or sVCAM-1 molecules, to support the physiological prosecution of pregnancy. Consequently, we performed a mediation-moderation analysis to investigate associations between different EV types and levels of the proinflammatory sICAM-1 or sVCAM-1 molecules and observed that long-term PM_{10} exposure is linked to increased Sync-1 + EV and decreased sVCAM-1 concentrations. This evidence suggests that the inflammatory response induced by PM might be mitigated in pregnant women by the release of placenta-derived EVs that exert a protective role.

We recently reported in the INSIDE population that both short-term and long-term exposures to PM_{10} were associated with increased levels of maternal VCAM-1, a cell adhesion molecule, and a marker of endothelial activation (Mozzoni et al. 2022). Consistently with our findings, exposure to air pollutants has been previously associated with higher levels of VCAM-1 and, in turn, with an increased risk for cardiovascular outcomes, both in healthy and diseased people (Alexeeff et al. 2011;

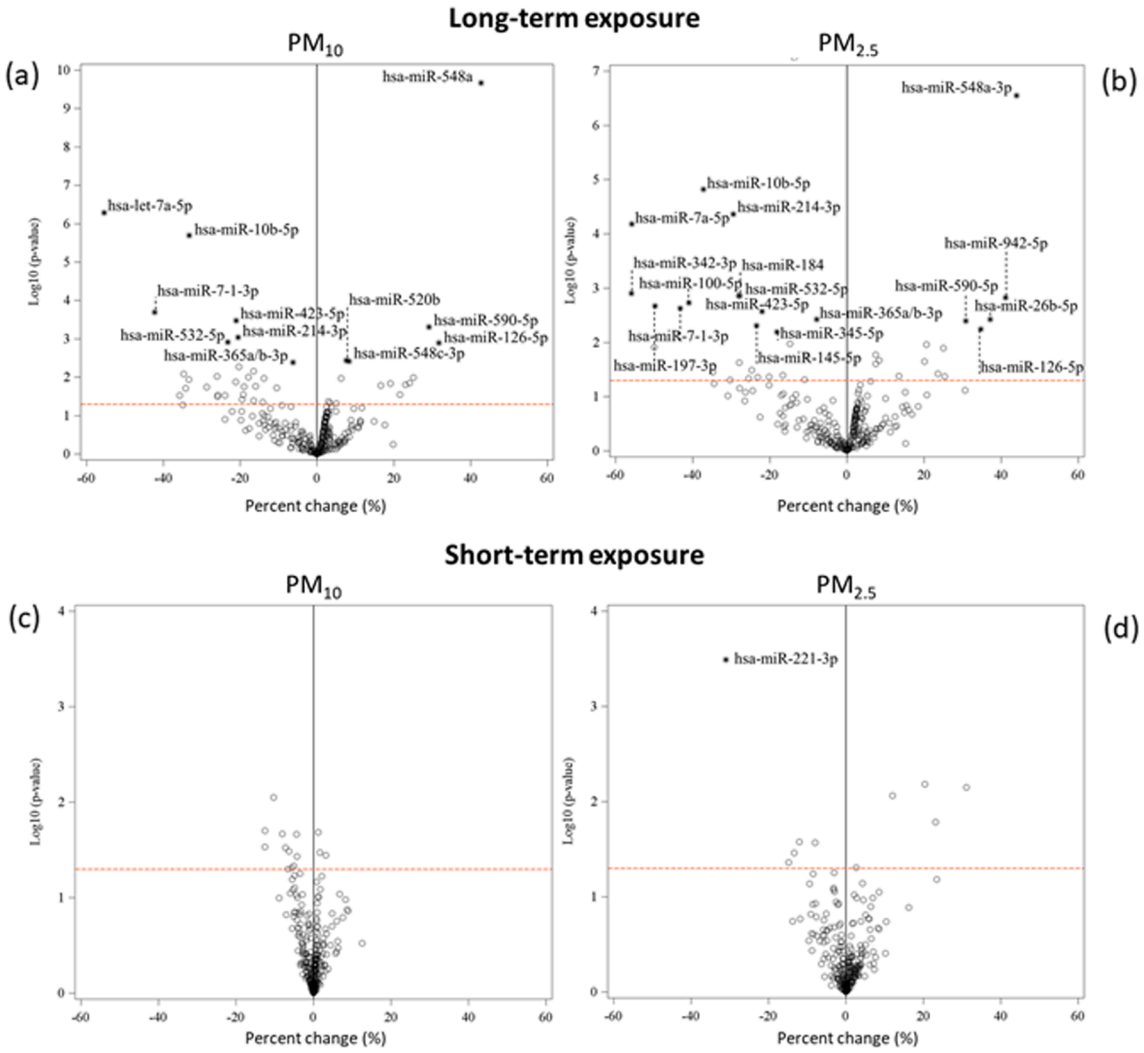

Fig. 3. miRNA expression after long- and short-term exposure to PM. (a) Long term exposure to PM_{10}; (b) long-term exposure to $PM_{2.5}$; (c) short-term exposure to PM_{10}; (d) short-term exposure to $PM_{2.5}$. Volcano plot shows on the Y-axis the $-\log_{10}$ of the p-value and X-axis the percent change for 10 μg/m^3 increment in exposure calculated from multivariable linear regression models including both long- and short-term effects of PM. Models were adjusted for age, gestational age at recruitment, BMI, smoking habits, temperature, and relative humidity. The percentage of change and the relative confidence interval were calculated using the formula [(exp(β-1))*100]. The dashed red line shows where $p = 0.05$. Points above the line have $p < 0.05$ and points below the line have $p > 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Yatera et al. 2008). On the contrary, we did not observe any associations for sICAM-1, and literature is inconsistent in this field. ICAM-1 is a cell adhesion molecule constitutively expressed at low levels by leukocytes and endothelial cells, and whose expression can be enhanced upon cytokine stimulation. On the other hand, VCAM-1 is expressed by vascular endothelial cells only after cytokine stimulation (Cook-Mills et al. 2011; Kong et al. 2018; Videm and Albrigtsen 2008). Thus, we speculate that while PM exposure may influence VCAM-1 expression and the release of its soluble form sVCAM-1, it might not directly affect ICAM-1 expression, or at least the release of its soluble form sICAM-1.

As we considered the long-term effects of PM exposure on EV-packaged miRNAs, we identified thirteen EV-miRNAs that were down-regulated and seven that were upregulated. All showed putative roles in inflammation, gestational hypertension, and pre-eclampsia. After miRNA-target interaction analysis, we further identified twelve genes involved in all the three-abovementioned processes. In particular, the *ADM* gene encodes adrenomedullin, a biopeptide hormone that is involved in angiogenesis and inflammation, and its altered plasmatic concentrations are associated with pre-eclampsia (Matson and Caron 2014), as well as angiotensinogen (encoded by *AGT*), which is one of the most studied molecules involved in blood pressure regulation (Gathiram and Moodley 2020; Shu et al. 2021). *SERPIN1* encodes a serine proteinase inhibitor of fibrinolysis, and its increased expression is associated with abnormal placentation and various reproductive diseases (Ye et al. 2017). Apelin (encoded by *APLN*) is secreted by the placenta during pregnancy, and exerts several cardiovascular functions (Wysocka et al. 2018); its serum and coronary capillary levels are decreased in pre-eclampsia (Inuzuka et al. 2013; Leeper et al. 2009; O'Carroll et al. 2013). Endothelin-1 (encoded by the *EDN1* gene) is an endothelium-secreted peptide, with a recently reported pathophysiological role in primary placental disorders and the systemic endothelial dysfunction of pre-eclampsia (Granger et al. 2018). Fms-like tyrosine kinase-1 (*FLT1*) is an antiangiogenic protein produced by the placenta that plays an important role in embryonic angiogenesis and vasculogenesis, and that can inhibit the activities of both vascular endothelial growth factor-A (VEGFA) and placental growth factor (PlGF), inducing symptoms of pre-eclampsia in animal models (Andraweera et al. 2012). Moreover, clinical studies showed that the soluble-FLT1 to PlGF ratio could predict pre-eclampsia (Nikuei et al. 2020). Dysregulation of cytokines *IL6* and *IL10* has also been linked to pregnancy complications associated with the cardiometabolic health of the mother and the newborn, including pre-eclampsia and preterm birth (Zak and Soucek 2019). Leptin (*LEP*)

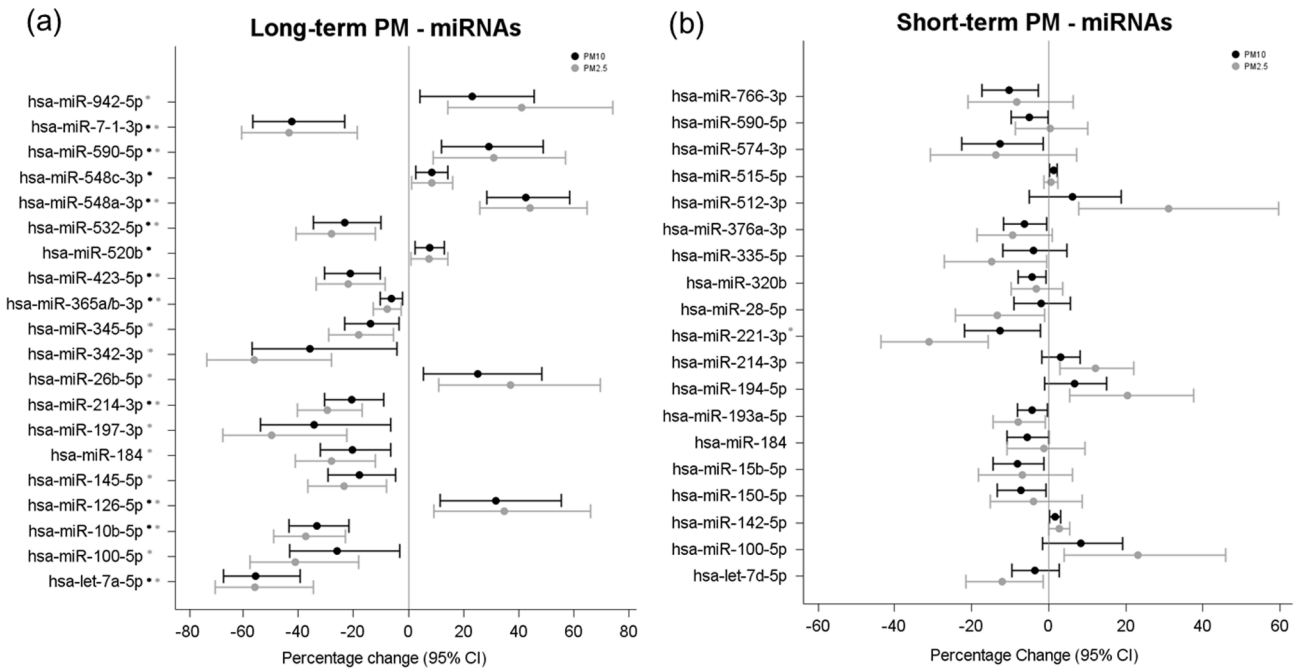

Fig. 4. .iRNA expression changes after long-term exposure to PM_{10} (panel a) and $PM_{2.5}$ (panel b). Percent change of the 20 miRNAs that were altered in response to long-term exposure to PM. Asterisks indicate PM-miRNA associations which qFDR < 0.10, where the qFDR is the adjusted p-value taking into account the false discovery rate. Black asterisks refer to PM_{10}; grey asterisks refer to $PM_{2.5}$.

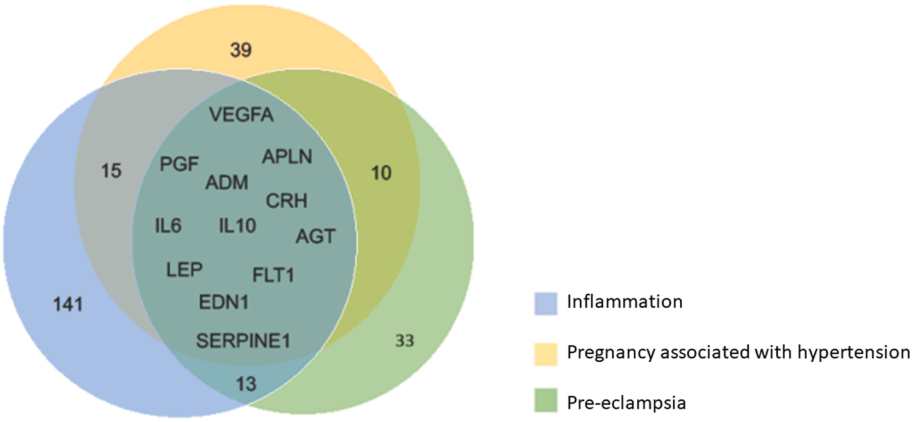

Fig. 5. miRNA target analysis. Venn diagram reports the 12 gene targets involved in inflammation and pregnancy associated with hypertension and pre-eclampsia. The selected genes were targeted by the 21 miRNAs associated with long- and short- term PM_{10} and $PM_{2.5}$ exposures.

regulates multiple aspects of maternal metabolic homeostasis such as placentation, maternal-fetal exchanges, and normal fetal growth; its pro-inflammatory action promotes the pathogenesis of various complications of pregnancy (Perez-Perez et al. 2018). Corticotropin-releasing hormone (CRH) is released into maternal and fetal circulations from the placenta; elevated concentrations are associated with pregnancy-related conditions (Davis et al. 2005).

Only one miRNA (hsa-miR-221-3p) was downregulated after short-term PM exposure. Interestingly, a previous study reported a down-regulation of miR-221-3p in pre-eclampsia (Yang et al. 2019). Moreover, in vitro functional assays showed that miR-221-3p expression promoted trophoblast growth, invasion, and migration; consequently, these biological processes might be affected by miR-221-3p regulation.

This study has several strengths. First, the study population was characterized by a large sample size and we devoted particular attention to methodological issues. The present study was conducted taking into account the International Society for Extracellular Vesicle minimal technical requirements in every step of the investigation, from sample collection to EV isolation and characterization. Second, NTA and flow cytometry were performed on fresh blood, to avoid hemolysis and to limit EV modifications (Yuana et al. 2011). Third, the analyses were performed on women who were followed until the end of pregnancy, thus resulting in a well-characterized clinical assessment.

We also acknowledge some limitations. First, the study population included only healthy women, and thus did not consider women with pregnancy-related risk factors at recruitment (cancer, cardiovascular diseases, or other chronic diseases such as diabetes, multiple sclerosis, epilepsy, schizophrenia, and depression). Therefore, the INSIDE population may not be completely representative of the general population. Second, we estimated PM exposures at the participants' residential addresses, without considering their daily actual activities and locations (i.e., personal sampling), nor the indoor PM levels, which may have resulted in misclassifications of the exposure attributions. Third, plasma samples were obtained during the first trimester of gestation, thus other processes influencing pregnancy outcomes might occur in the later stages after PM exposures. Moreover, EV-miRNAs were evaluated after

the extraction of miRNAs from the whole plasmatic EV population and did not focus on specific EV types. Future studies should be conducted on specific EV populations, to better decode the molecular signals transmitted between different cell types.

5. Conclusion

Taken together, our findings strongly support the hypothesis that EVs have an important role in modulating the effects of PM exposure during pregnancy. In particular, Sync + EVs, released primarily from the placenta, might counterbalance the proinflammatory effect of PM exposure. Moreover, EV action is possibly exerted through their miRNA cargo.

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7. Authors' contributions

LF performed miRNA expression analysis, contributed to data analysis and interpretation, and wrote the manuscript; SI performed statistical analysis; LC performed EV analysis; GS performed bioinformatic analysis; LD contributed to plasma separation and miRNA analysis; MH performed EV analysis; MV contributed to the enrolment of subjects; PM performed sICAM-1 and sVCAM-1 analysis; EB supervised the study and sICAM-1 and sVCAm-1 analysis; NP supervised the study and enrolled the pregnant subjects; VB supervised the whole study, received funding and supervised the writing of the draft. All authors approved the final version of the submitted version.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Luca Ferrari performed miRNA expression analysis, contributed to data analysis and interpretation, and wrote the manuscript; Simona Iodice performed statistical analysis; Laura Cantone performed EV analysis; Giulia Solazzo performed bioinformatic analysis; Laura Dioni contributed to plasma separation and miRNA analysis; Mirjam Hoxha performed EV analysis; Marco Vicenzi contributed to the enrolment of subjects; Paola Mozzoni performed sICAM-1 and sVCAM-1 analysis; Enrico Bergamaschi supervised the study and sICAM-1 and sVCAm-1 analysis; Nicola Persico supervised the study and enrolled the pregnant subjects; Valentina Bollati supervised the whole study, received funding and supervised the writing of the draft.

All authors approved the final version of the submitted revised version.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107502.

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