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Research Article

EV-miRNA associated with environmental air pollution exposures in the MADRES cohort

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Abstract

Air pollution is a hazardous contaminant, exposure to which has substantial consequences for health during critical periods, such as pregnancy. MicroRNA (miRNA) is an epigenetic mechanism that modulates transcriptome responses to the environment and has been found to change in reaction to air pollution exposure. The data are limited regarding extracellular-vesicle (EV) miRNA variation associated with air pollution exposure during pregnancy and in susceptible populations who may be disproportionately exposed. This study aimed to identify EV-miRNA expression associated with ambient, residential exposure to $PM_{2.5}$, PM_{10}, NO_2, O_3 and with traffic-related NO_x in 461 participants of the MADRES cohort, a low income, predominantly Hispanic pregnancy cohort based in Los Angeles, CA. This study used residence-based modeled air pollution data as well as Nanostring panels for EVmiRNA extracted with Qiagen exoRNeasy kits to evaluate 483 miRNA in plasma in early and late pregnancy. Average air pollution exposures were considered separately for 1-day, 1-week, and 8-week windows before blood collection in both early and late pregnancy. This study identified 63 and 66 EV-miRNA significantly associated with $PM_{2.5}$ and PM_{10}, respectively, and 2 miRNA associated with traffic-related NO_X (False Discovery Rate-adjusted P-value $< .05$). Of 103 unique EV-miRNA associated with PM, 92% were associated with lung conditions according to HMDD (Human miRNA Disease Database) evidence. In particular, EV-miRNA previously identified with air pollution exposure also associated with $PM_{2.5}$ and PM_{10} in this study were: miR-126, miR-16-5p, miR-187-3p, miR200b-3p, miR486-3p, and miR-582-3p. There were no significant differences in average exposures in early vs late pregnancy. Significant EV-miRNAs were only identified in late pregnancy with an 8-week exposure window, suggesting a vulnerable timeframe of exposure, rather than an acute response. These results describe a wide array of EV-miRNA for which expression is affected by PM exposure and may be in part mediating the biological response to ambient air pollution, with potential for health implications in pregnant women and their children.

Keywords: microRNA; air pollution; pregnancy; prenatal environment; particulate matter

Introduction

Globally, air pollution is a major health hazard: the World Health Organization describes air pollution as the world's largest environmental risk factor for disease [1], comparable to well-established risks like unhealthy diets and tobacco smoking [2]. More than 3.5 million deaths per year are attributable to ambient air pollution including those via heart disease, stroke, and respiratory complications [1]. Ambient air pollution has a number of different components with different risks and hazardous effects. These mixtures include gases such as ozone (O_3), nitrogen dioxide (NO_2), nitrogen oxides (NO_x), sulfur dioxide, and particulate matter (PM), as well as other chemicals and smoke components [3].

Particulate matter air pollution can vary in size distribution, and $PM_{2.5}$ and PM_{10} or particles with aerodynamic diameter $< 2.5\,\mu m$ or $10\,\mu m$, respectively, are regulated as criteria air pollutants [4]. These particles consist of different components, including metals, organic compounds, hydrocarbons, and ions. Globally, exposure to $PM_{2.5}$ and PM_{10} are known to contribute to heart disease, stroke, chronic obstructive pulmonary disease (COPD), diabetes mellitus, and lung cancer [2, 5–9].

Exposure to outdoor air pollution is also a hazard during pregnancy and could negatively impact children's future health. Prenatal exposure to $PM_{2.5}$ and PM_{10} is associated with reduced fetal growth, miscarriage, stillbirth, and preterm birth, especially for

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male infants and children in locations with lower socioeconomic development [10–13]. Ambient air pollution is also associated with postpartum depression [14], gestational diabetes mellitus (GDM) [15, 16], which can lead to pregnancy complications, increase later health risks for the child [17], and signal metabolic syndrome risk for the mother [18]. Prenatal air pollution is also associated with neonatal complications [19], childhood asthma [20], and obesity [21, 22] and is also associated with mental health disorders in adolescence [23]. These negative effects of air pollution are suspected to carry down through generations and will perpetuate health disparities among those exposed to high pollution and at greater vulnerability to its effects [24]. Specifically, individuals living in environmental justice communities are disproportionately exposed to air pollution from local toxic sources such as traffic and industry as a result of long-term discriminatory practices and policies, and they are also at greater risk or vulnerability to its adverse effects [25–27].

Many mechanisms have been proposed to explain the biological effects of air pollution, including inflammatory pathways and oxidative stress [28, 29]. Biomarkers of different health responses to air pollutant exposures might be useful for clinicians and public health professionals, particularly to help identify vulnerable periods of time, or potentially to inform follow-up for certain disease risks over time to which those biomarkers are related. Several biomarkers have been proposed for physiological effects or pathways of response to air pollution exposure, such as C-reactive proteins, placental DNA methylation, and mitochondrial DNA methylation, all of which have been observed to change with acute and/or chronic exposure to air pollution [30].

MiRNA are another potential epigenetic biomarker. MiRNA are short segments of RNA (~22 nt) that modulate transcriptome expression and protein translation and have been observed to change with environmental exposures [31–33]. In addition to acting within the cell where they are produced, miRNA are also packed into extracellular vesicles and move through circulation to target tissues throughout the body, and through this mechanism may explain systemic biological responses to air pollution exposure and play a substantial role in pregnancy [34–37]. Candidate miRNA for air pollution biomarkers have been suggested, including miR-222, miR126-3p, and miR-200c that are also associated with lung cancer risk [38], as well as miR-200b, let-7c, and miR-378d in breastmilk [39], and miR-30b and c, miR10b, and miR181a in plasma [40]. Clinicians and public health researchers could identify and monitor at-risk individuals over time using informative miRNA biomarkers for the effects of air pollution exposure given their accessibility and responsiveness.

A thorough review of the literature for miRNA and air pollution by Sima *et al.* [38] compiled a number of EV-miRNA associated with different air pollutants and considered the effects in sensitive populations [38] (e.g. those with occupational exposures, children, the elderly, etc.), but did not contain much detail on pregnant women and prenatal exposures, nor health disparities groups, demonstrating a clear need for further research.

Hypothesis and objectives

This study aims to identify EV-miRNA associated with residential exposures to ambient and traffic-related air pollution among pregnant women in an under-resourced cohort. We hypothesize that some miRNA will be significantly associated with higher exposure to air pollution in 1-day, 1-week, or 8-week intervals preceding blood and miRNA collection. Additionally, some of these miRNA may be shared between different types of air pollution.

Table 1. Demographic table of participants assessed for miRNA and air pollution exposure

		miRNA mothers	
		n	**Mean (SD)**
Maternal age	At 12 weeks of pregnancy	461	28.5 (5.9) years
miRNA samples timing	Early pregnancy	267	13.2 (4.2) weeks
	Late pregnancy	395	31.6 (2.0) weeks
		n	**Percent**
GA at birth	Preterm (<37 weeks)	45	9.8
	Term (37–40 weeks)	292	63.3
	Late term (40–42 weeks)	124	26.9
Fetal sex	Female	227	49.2
	Male	233	50.5
Parity	Nulliparous	146	31.7
	Primiparous or higher	297	64.4
Language	English	298	64.6
	Spanish	163	35.4
Race/ethnicity	US-born White Hispanic	162	35.1
	Foreign-born White Hispanic	190	41.2
	Other Hispanic	27	5.9
	Black Non-Hispanic	49	10.6
	Non-Hispanic other	28	6.1
	Unknown or not reported	5	1.1
Education	Less than 12th grade (did not finish high school)	127	27.5
	Completed 12th grade (graduated high school)	136	29.5
	Some college or completed college	193	41.9
Pre-pregnancy BMI	Underweight or Normal Weight (<25 kg/m^2)	125	27.1
	Overweight (25 kg/m^2–29.9 kg/m^2)	157	34.1
	Class 1–3 obese (>30 kg/m^2)	168	36.4

Notes: Samples may not add to 100% due to missingness in questionnaires. GA at birth may be unavailable for participants lost to follow-up at birth; however, the best estimated GA during pregnancy was available, as described in the "Methods" section.

Results

Cohort

Participants in this study were generally reproductive age women with a mean age of 28.5 ±6 years (for 461 participants, Table 1). Gestational age (GA) at delivery was mostly at term and late-term (63% and 27%, respectively), with only 10% delivering before 37 weeks. Approximately 76% identified as Hispanic and 10% identified as Black. Just over 30% of participants were in their first pregnancy, while 64% had at least one previous pregnancy. This population had high levels of overweight and obese mothers (34% and 36%, respectively), with only 27% normal or underweight prior to pregnancy and just 10 participants reported smoking during pregnancy (2.2%).

Air pollution

Average air pollution values for the participants in this study ranged widely (Table 2) but were generally correlated (SI Fig. S1 in supplementary material), with mean (SD) values for 1 week windows in early pregnancy were 16.3 (7.5) ppb for NO_2, 27.5 (7.5) ppb for O_3, 12.0 (4.7) $\mu g/m^3$ for $PM_{2.5}$, 30.2 (8.8) $\mu g/m^3$ for PM_{10}, and 3.6 (1.7) ppb for traffic NO_x. Average air pollution values for all windows (1-day, 1-week, and 8-week) were largely similar. There was no significant difference for average participant pollutant values

Table 2. Air pollution concentrations summary statistics for early and late pregnancy and for 1- and 8-week exposure windows

Stage	Window	Pollutant	Mean	SD
Early pregnancy	1 day	NO_2 (ppb)	16.41	8.3
		O_3 (ppb)	27.28	8.0
		$PM_{2.5}$ ($\mu g/m^3$)	11.66	5.07
		PM_{10} $\mu g/m^3$)	30.15	10.91
		Traffic NO_x (ppb)	3.86	2.53
	1 week	NO_2	16.3	7.5
		O_3	27.5	7.5
		$PM_{2.5}$	12.0	4.7
		PM_{10}	30.2	8.8
		Traffic NO_x	3.6	1.7
	8 weeks	NO_2	16.3	6.4
		O_3	27.0	6.7
		$PM_{2.5}$	12.0	2.8
		PM_{10}	29.8	6.0
		Traffic NO_x	3.6	1.6
Late pregnancy	1 day	NO_2	17.46	8.15
		O_3	25.34	8.67
		$PM_{2.5}$	12.05	6.27
		PM_{10}	28.83	10.39
		Traffic NO_x	3.83	2.64
	1 week	NO_2	17.5	7.0
		O_3	25.5	8.2
		$PM_{2.5}$	12.4	4.9
		PM_{10}	29.3	9.0
		Traffic NO_x	3.8	1.8
	8 weeks	NO_2	17.0	6.1
		O_3	26.1	7.1
		$PM_{2.5}$	12.3	2.5
		PM_{10}	29.6	6.1
		Traffic NO_x	3.6	1.8

for NO_2, traffic NO_x, $PM_{2.5}$, and PM_{10} across timepoints and exposure average windows (Analysis of Variance, $P > .05$). There was a significant difference between average exposures of O_3 between the early and late pregnancy for the 1-week exposure window (T-test, $P = .001$).

Air pollution-associated miRNA

$PM_{2.5}$ and PM_{10} were significantly associated with the differential expression of 63 and 66 miRNA, respectively, in late pregnancy for the 8-week exposure window (Table 3). Of those, 23 miRNA shared increased expression for both exposures. Effect estimates were scaled to percent change in EV-miRNA count per IQR change in pollutant, ranging from −9.4 to 16.1, with a mean of 10.3 for $PM_{2.5}$ and ranging from −19.7 to 42.2 with a mean of 15.7 for PM_{10}. Of these significantly differentially expressed EV-miRNA, 59 EV-miRNA were upregulated and 4 were downregulated for $PM_{2.5}$ exposure and 65 upregulated and 1 downregulated for PM_{10} exposure (Selected miRNA: Fig. 1, Volcano plot: Fig. 2, Complete list: SI Tables S2 and S3 in supplementary material). Fourteen additional miRNA had differential expression levels suggestively associated with $PM_{2.5}$ exposure at the early pregnancy timepoint considering the previous 8 week of exposure (FDR $P < .1$). No miRNAs were significantly associated with O_3, NO_2, or NO_x for any of the three windows considered for the late pregnancy timepoint. No EV-miRNAs were differentially expressed after adjustment for the shorter 1-week and 1-day exposure windows in early pregnancy, except for two miRNAs positively associated with acute 1-day exposure to NOx in early pregnancy (miR-1537-3p and mir-585-3p).

Table 3. Overview of results: number of miRNA significantly associated with each pollutant for three exposure windows in early and late pregnancy (FDR-adjusted $P < .05$)

		Exposure window		
	Pollutant	1 day	1 week	8 weeks
Late pregnancy	**$PM_{2.5}$**	0	0	**63**
	PM_{10}	0	0	**66**
	NO_2	0	0	0
	O_3	0	0	0
	NO_x	0	0	0
Early pregnancy	$PM_{2.5}$	0	0	0
	PM_{10}	0	0	0
	NO_2	0	0	0
	O_3	0	0	0
	NO_x	**2**	0	0

Sensitivity analyses excluding participants reporting smoking showed that all 63 miRNAs associated with $PM_{2.5}$ remained significant, but just 22 of the 66 miRNAs associated with PM_{10} remained significant (SI Table S4 in supplementary material). Additional validation by permutation showed no miRNAs were significantly associated with PM or NO_X after air pollution values were permuted among participants, suggesting no or limited spurious associations in the main analysis. Additional sensitivity analysis with seasonal variables (season of sample, relative humidity, and average daily temperature) as both covariate adjustment and effect modifier showed that 39 of the 66 miRNA associated with PM_{10} remained significant after FDR correction, but none of the miRNA associated with $PM_{2.5}$ remained statistically significant. Season, rather than humidity or temperature, drove these effects. Models including an interaction term between season and pollution for $PM_{2.5}$ found that the effects of $PM_{2.5}$ were strongest in spring for 61 miRNAs and statistically significant for 6 miRNAs, including miR-1249-3p, miR-1302, miR-337-5p, miR-548k, miR-548 v, and miR-891a-5p.

Pathway analysis

In total, 106 unique EV-miRNA that were significantly differentially expressed in relation to PM_{10} and/or $PM_{2.5}$ exposures were then assessed for association with known diseases using HMDD and miTED, a tissue expression atlas. Ninety-eight of the 106 unique EV-miRNAs (92.4%) were enriched for those miRNA previously linked with lung conditions in HMDD (SI Table S3 in supplementary material). Forty-four of these EV-miRNA were also enriched among miRNA related to at least one of the other HMDD categories: cardiovascular, neuroendocrine, depression, and inflammation. Gene targets of these miRNA were categorized into 22 sets of PANTHER pathways (Fig. 3), including epidermal growth factor (EGFR) and vascular endothelial growth factor signaling pathways, insulin/Insulin-like growth factor (IGF), and p53-related pathways. Similar pathways were found for miRNA associated with both PM_{10} and $PM_{2.5}$ (SI Table S5 in supplementary material), including p53 and p38MAPK, insulin-IGF, and EGFR pathways.

Using the miTED tissue atlas, 37 of the 63 (59%) miRNA associated with $PM_{2.5}$ were expressed in lung, while 35 of the 66 miRNA (53%) associated with PM_{10} were expressed in lung. Likewise, 43 of the 63 (68%) miRNA associated with $PM_{2.5}$ were expressed in placenta, and 41 of the 66 miRNA (62%) associated with PM_{10} were expressed in placenta.

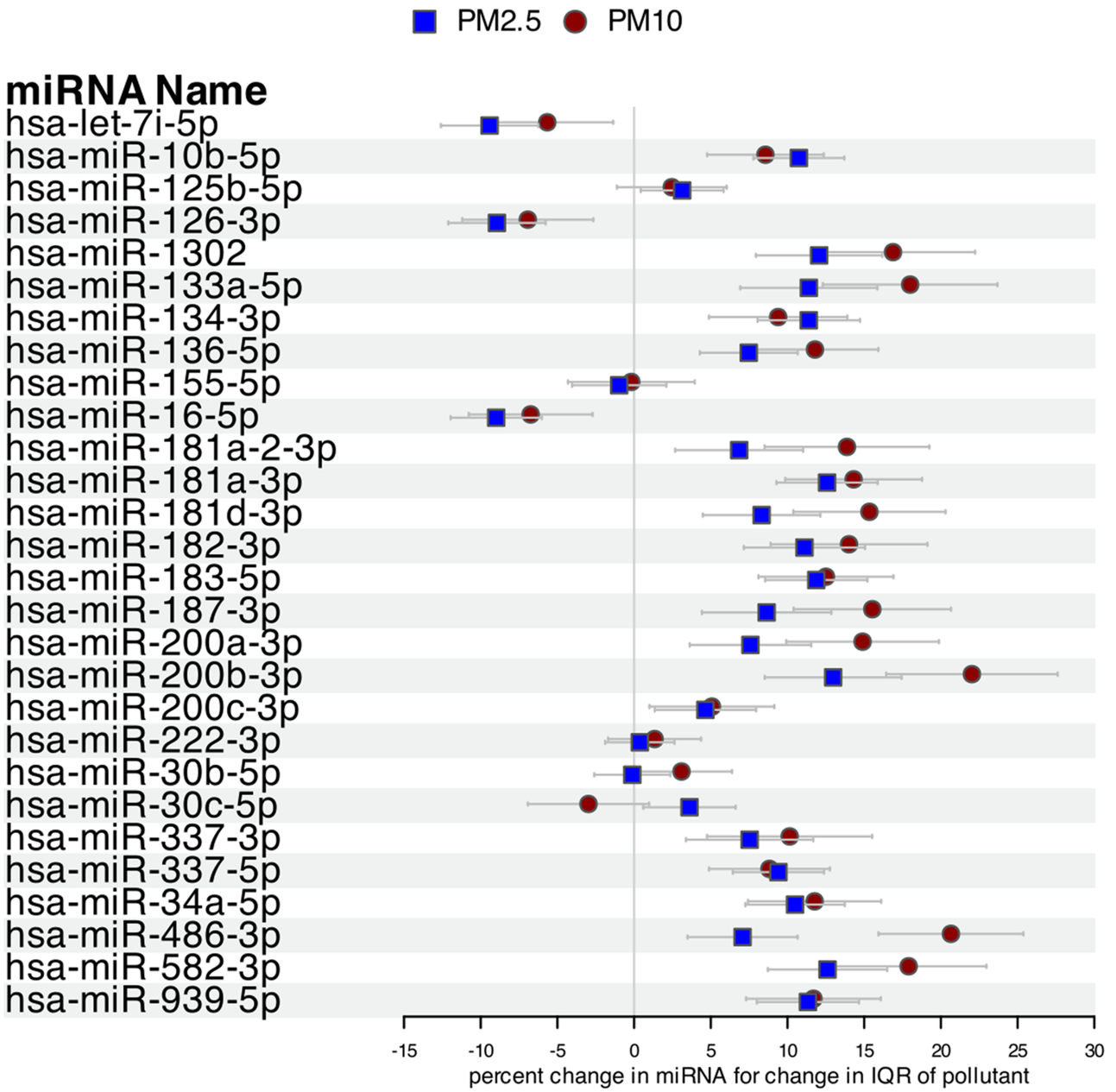

Figure 1. Forest plot of selected miRNA estimates significantly associated with air pollution after FDR adjustment (scaled to percent change in miRNA counts for IQR change in pollution exposure), considering an 8-week exposure window before late pregnancy blood sample (31.6 ± 2.0 weeks) (Full list in SI Tables S2, S3).

Discussion

This study identified 106 circulating EV-miRNA whose differential expression was associated with particulate matter air pollution in late pregnancy in a subgroup of MADRES participants. Importantly, associations were observed only for the longer exposure window of 8-week and not acute 1-week exposures. These effects were also limited to late pregnancy exposure periods and most miRNAs were positively associated with exposure to PM. The bias of miRNA positively associated with PM exposure may reflect changes to the intracellular miRNA landscape and may be selectively packaged into EVs, as seen in diabetes mellitus and chronic lung disease [41]. Twenty-three of the miRNAs were associated with both PM_{10} and $PM_{2.5}$, so that nearly 30% of the miRNAs were shared between exposures. These PM-associated EV-miRNAs were associated with various conditions, including neuroendocrine, inflammation, cardiovascular, and were particularly enriched in healthy lung expression (53%–58% of miRNA) in miTED data and in associations with lung conditions (92% of miRNA) in HMDD data.

The PM-associated EV-miRNA identified in this study have also been implicated in pregnancy complications, especially via inflammatory pathways. Gene targets of the miRNA shared between $PM_{2.5}$ and PM_{10} included several inflammatory pathways, including mitogen activated protein kinase (MAPK), T-cell activation, and Ras pathways. miR-200b, miR-182-3p, and miR-187 were significantly positively differentially expressed with $PM_{2.5}$ and PM_{10} in our study, consistent with previous studies that found increasing levels of these miRNA are tied to preeclampsia [42–44].

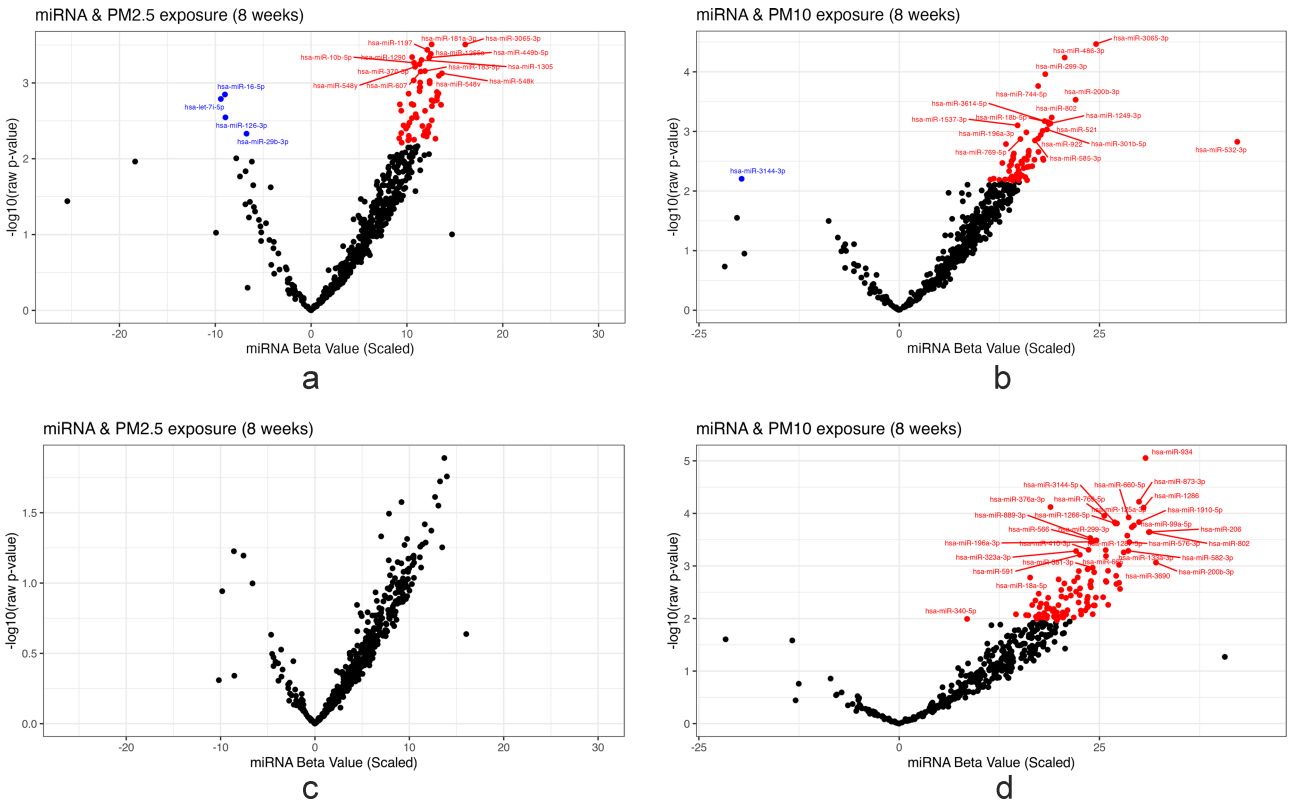

Figure 2. Volcano plots of miRNA significantly associated (FDR<0.05, red: upregulated, blue: downregulated) with (a) PM2.5: *N*=63. (b) PM10: *N*=66. (c) PM2.5 with season interaction: *N*=0. (d) PM10 with season interaction: *N*=39.

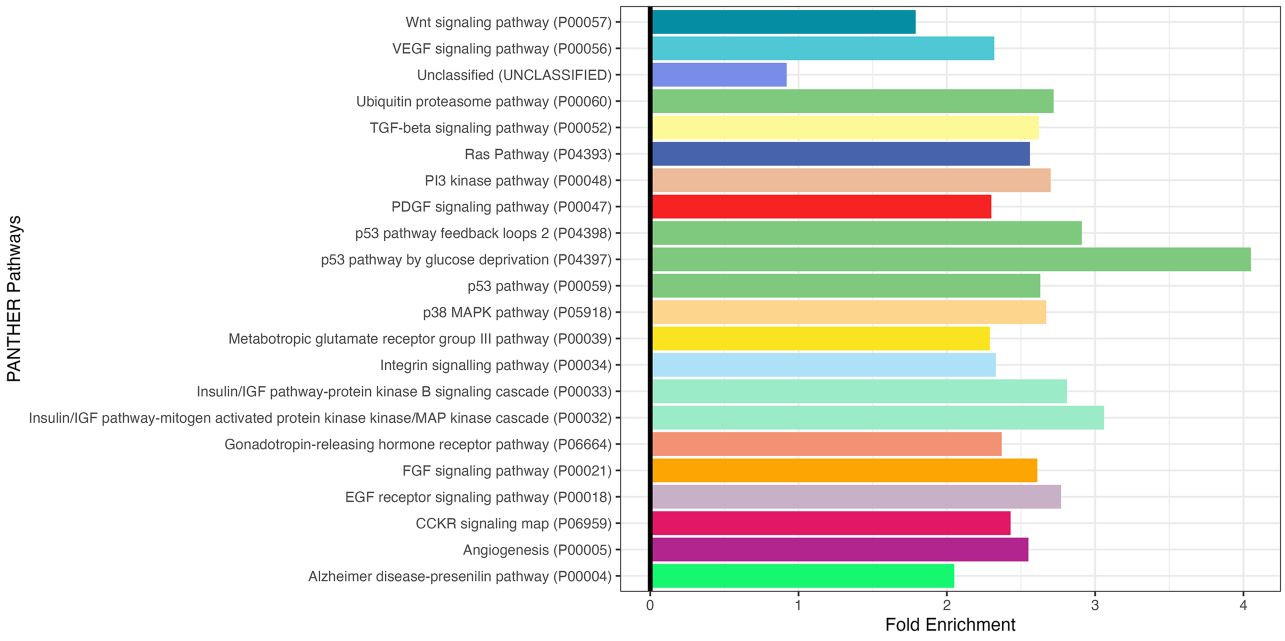

Figure 3. PANTHER pathways of gene targets from miRNA significantly associated with PM10 and PM2.5 and associated with lung conditions (HMDD).

In similar roles, miR-16-5p and miR-200b are associated with trophoblastic invasion of the placenta [42–46], which is critical to developing a healthy placenta and preventing pre-eclampsia [47, 48]. Increased levels of these two miRNA in placentas are associated with decreased cell migration and invasion and/or pre-eclampsia [49, 50]; however, EV-associated miR-126-3p and miR16-5p were negatively differentially expressed with exposure to $PM_{2.5}$ and PM_{10}. Decreased levels of miR-136-5p have also been associated with preterm labor [51]. Eleven of the miRNA exhibiting differential expression associated with PM_{10} or $PM_{2.5}$ have been identified in pregnancy-specific and/or placental expression [52]: let-7i-5p, miR-126-3p, miR-1302, miR-134-3p, miR-181a-3p,

miR-200b-3p, miR-34a-5p, miR-133a-5p, miR-136-5p, miR-181d-3p, and miR-200a-3p. These miRNAs are known to be part of the pregnancy-related miRNA clusters on chromosomes 14 and 19 and are commonly detected in the placenta, as well as in maternal circulation [53–55]. Taken together, these results suggest that EV-miRNA modulated by air pollution may also play a role in pregnancy complications.

EV-miRNA may play critical roles in the defensive response to PM air pollution exposure such as acute inflammatory responses. Decreased levels of miR-187-3p have been previously associated with increased $PM_{2.5}$, and act on tumor necrosis factor-α, a cytokine associated with acute inflammatory responses in macrophages [56]; however, we observed increased miR-187-3p expression with increasing PM_{10} exposure. Another protective miRNA, miR-486, inhibits apoptosis and limits oxidative stress as a result of $PM_{2.5}$ exposure, suggesting that miRNA activity is part of a normal defensive response [57]. Other EV-miRNA associated with air pollution here may be expressed in placenta tissue: PM_{10} modulates miR-125b-5p expression from placental trophoblast cells *in vitro* [58], and may play a slightly different pregnancy-specific role compared to previous studies. While this paper did not identify miRNA associated with NO_2 or O_3, these pollutants are also known to affect circulating miRNA, including let-7i-5p, miR-125b-5p, and miR-126-3p [59–61], which were also identified in this paper as associated with PM. Another miRNA identified in this study, miR-200b-3p, has also been suggested as a biomarker for air pollution exposure during pregnancy via EV-miRNA in breastmilk [39]. More generally, circulating miRNAs have previously been proposed as biomarkers in complications of pregnancy including preterm birth [62] and pre-eclampsia [63], and those miRNAs associated with both pollution exposure and complications may warrant further research.

A number of the observed pollution-associated EV-miRNA have previously been associated with environmental exposures. Exposure to diesel exhaust has been associated with changes in miRNA levels for miR-16-5p and miR-183-5p, in synergy with allergen exposures [61, 64] and both sulfur dioxide (SO_2) and $PM_{2.5}$ were found to be associated with miR-337, which may be involved in tau phosphorylation leading to neurodegenerative effects [65]. Wildfire smoke exposure, while different than the ambient and traffic-related exposures studied here, has also been observed to alter the expression of PM-associated miRNA found in this study, especially miR-126 and miR-155 [66]. miR-126 has been suggested as a biomarker for air pollution-related cancer risk due to its detection in plasma and serum across lung cancer studies [38]. $PM_{2.5}$ and PM_{10} as pollutants are in and of themselves considered mixtures of multiple chemical components, and their composition can vary drastically based on the sources from which they originated. In our study region, $PM_{2.5}$ is largely impacted by traffic, industry, and secondary formation, while PM_{10} is heavily impacted by dust resuspension, marine aerosols, crustal materials, road dust, and more, and both can be impacted by wildfire smoke [67]. Interestingly, in a sensitivity analysis, all the miRNA associated with $PM_{2.5}$ retained significance when smokers were excluded, while 44 of the 66 miRNAs associated with PM_{10} lost significance. Therefore, future research into the specificity and utility of select miRNA as biomarkers of pollution-induced health risk, and in particular more source resolved or specific pollutant signatures, might be particularly useful as a tool in health risk assessment particularly in high-risk populations.

When including season of sample collection, relative humidity and temperature, the miRNA associated with $PM_{2.5}$ were attenuated, while 39 of the 66 miRNAs associated with PM_{10} were retained. Seasonal environmental variables were not available for all participants, which reduced our sample size by up to 38 participants and may have also reduced the power of models including the seasonal covariates. Much of the reduction in effect size was driven by season of collection: however, models including an interaction term between $PM_{2.5}$ and season showed that the pollution effect was greatest in the spring for most of the miRNA (61 of 63 miRNAs significantly associated with $PM_{2.5}$ in the main analysis, SI Table S6 in supplementary material) thus illustrating the importance of season as a modifying factor. Effect sizes were largely the same between the main analysis for models with relative temperature or relative humidity, with little change in the number of miRNA significantly associated with $PM_{2.5}$. These data underscore the complex associations between different size fractions of particulate matter and how they are affected by environmental and meteorological variables. Given the relative importance of the spring season for $PM_{2.5}$, for instance, it will be important to further explore potential variation in sources of $PM_{2.5}$ as well as the presence and potential interacting effects of airborne allergens.

While not identical to outdoor air pollution, further evidence for the role of miRNA can be drawn from studies of tobacco smoke that share many particulate and gaseous pollutants. Prenatal exposure to tobacco smoke is a major risk factor for many health complications and continues the transmission of allergic asthma and bronchopulmonary dysplasia in part by miRNA also associated with $PM_{2.5}$ and PM_{10} in this study. miR16-5p and miR-939-5p, which target HIF-1α [20], have been observed to suppress endothelial injury due to $PM_{2.5}$ exposure *in vitro* [68]. miRNAs have also been suggested as a mediator in COPD and interstitial fibrosis and may play a similar role in exposure to tobacco smoke via airway inflammation and oxidative stress [69, 70]. Likewise, the overlap between miRNA associated with air pollution and those mediating lung cancer risk is substantial, suggesting that EV-miRNA may also mediate the effects of air pollution on lung cancer risk [38], asthma, COPD, and other lung diseases [71].

Downstream PANTHER pathways of the miRNA associated with PM included several associated with insulin signaling that suggests some of these miRNAs may be involved in both air pollution responses and metabolic processes, including insulin-related pathways. In MADRES, air pollution during sensitive windows in periconception and early pregnancy has previously been associated with higher risk of developing GDM [16]. Several oxidative stress-related pathways were also identified, including p53 pathways that have been associated with cellular stress responses [72], p38-MAPK pathways that have been associated with inflammation and senescence [73], and epidermal growth factor pathways that have been tied to lung inflammation [74]. As a whole, these pathways suggest that miRNAs are associated with an acute inflammatory response to PM exposure.

Despite identifying interesting inflammatory- and lung-associated miRNA, this study has a few limitations. Although the circulating miRNA in this study were derived from extracellular vesicles in blood plasma, we are not able to characterize the EV particles nor identify the tissue of origin for these EVs using immunoaffinity-based methods for EV isolation. Additionally, comparison to previously published data on tissue miRNA expression may yield different results than this paper's data on EV-miRNA due to differences in methods and limited re-validation of miRNA levels. However, the accessibility and ease of use of a commercially-available kit may be an advantage in widening the field and deploying clinical applications of miRNA in the future.

Fourteen EV-miRNAs were suggestively associated with $PM_{2.5}$ in early pregnancy (FDR $P < .1$), but analyses may be power-limited by the smaller sample size at this timepoint. Additionally, while outdoor air pollution estimates mainly captured regional or traffic-related local signals, and indoor sources of air pollution exposure, including secondhand smoke, were not considered, the dense monitoring network in southern California, combined with high resolution line dispersion modeling together provide good temporal and spatial resolution in important outdoor exposures in Los Angeles, CA. Sensitivity analyses excluding participants who reported smoking during pregnancy showed that the 63 miRNAs associated with $PM_{2.5}$ were retained, but 44 of the 66 miRNAs associated with PM_{10} lost significance. Future work on the sensitivity of miRNA to smoking and secondhand smoke during pregnancy may be relevant to maternal and fetal health. The data presented in this paper also does not address maternal or child outcomes associated with air pollution exposure, but as a longitudinal cohort, MADRES is well-situated to conduct such studies in the future as participants age.

Lastly, this study observed no significant difference between average daily PM exposures between first and third trimesters for either the 1-week or 8-week exposure window, although miRNAs were only significantly associated with air pollution for the late pregnancy 8-week window, suggesting that future research on critical windows of exposure later in pregnancy may have important influence on EV-miRNA expression and downstream health results.

Overall, these findings suggest that exposure to outdoor air pollution may be associated with upregulation of miRNA expression patterns in late pregnancy. These EV-miRNA play substantial roles in the lungs and inflammatory responses and may be key players mediating the downstream effects of air pollution exposure. Future studies may use these EV-miRNA as biomarkers to explore the relationship between pollution and pregnancy health. The current climate crisis is likely to intensify pollution [24], and it is critical to understand the effects of air pollution on miRNA and downstream pathways in pregnancy and child health while continuing to work toward a healthier climate.

Methods

Study design and summary

Using participant blood samples, miRNAs were isolated and quantified using Nanostring panels to identify those significantly associated with air pollution exposure. In the main analysis, the air pollution exposure levels were considered in 1-day, 1-week, or 8-week windows, while each miRNA was treated as an outcome. After adjusting for covariates, just over 100 miRNAs were significantly associated with at least one type of air pollutant evaluated. These miRNAs were investigated for downstream effects using several databases.

Cohort and participants

MADRES is a pregnancy cohort of more than 1000 women based in Los Angeles California (USA). Participants in this study are largely Hispanic and lower income [75]. Eligibility criteria were: (i) singleton pregnancy less than 30-week gestation, (ii) age 18 years or over, and (iii) able to speak English or Spanish. Participants were excluded if they were (i) HIV positive, (ii) currently incarcerated, (iii) carrying a multiple pregnancy, (iv) unable to participate or consent due to mental, physical, or cognitive disability. Written informed consent was obtained from each participant, and all procedures were approved by the institutional review board at the University of Southern California. A total of 461 participants (Table 1) with blood biospecimens were selected for this substudy. Criteria for inclusion were: (i) available blood biospecimens from early and/or late pregnancy during the study period (2015–2020), (ii) availability of pollution data, and (iii) availability of accurate GA at birth data, described in more detail below.

Demographic and study data were collected by staff-administered questionnaire, either by phone or in person. Among these questionnaires, the study staff identified each participant's language preference (English or Spanish), parity (number of previous pregnancies), level of maternal education (did not complete high school, completed high school, some college or higher), maternal age, race and ethnicity (Black non-Hispanic, non-Hispanic other, US-born Hispanic, foreign-born Hispanic, other Hispanic, unknown or not reported), and established pre-pregnancy BMI using staff-recorded height and early pregnancy or pre-pregnancy participant-reported weight. Maternal medical records were used to verify child's sex and GA at birth. GA at birth was determined using a hierarchy of methods: priority was given to first-trimester ultrasound measurement of crown-rump length; then, ultrasound measurement of fetal biparietal diameter in the second trimester; followed by obstetric clinical estimate abstracted from the participant's medical records; and lastly, by date of the participant's self-reported last menstrual period. GA at time of sample was calculated based on GA at birth and dates of collection, while maternal age was standardized at 12-week for all participants.

Four hundred sixty-one participants included in this study had one or two in-person visits [early (13.2 ± 4.2 weeks) and late (31.6 ± 2.0 weeks) pregnancy] at which anthropometric data (height, weight, and blood pressure) and blood specimens were collected. Blood samples were collected in EDTA tubes (BD #366643) and transported on ice to the MADRES laboratory. Within 2 h of blood draw, the EDTA tube was centrifuged at room temperature at 1300×*g* in swinging-bucket rotor (Beckman Coulter Allegra X-30 R or Avanti J-15 R) for 15 min to separate plasma, which was aliquoted in volumes of 500 μl in polypropylene cryovial tubes and frozen at −80°C until miRNA extraction.

EV-miRNA extraction

As described previously [32, 76], EV-miRNAs were extracted from thawed 500 μl plasma aliquots with the exoRNeasy kit (Qiagen) following the manufacturer's instructions. Briefly, extracellular vesicles were isolated and EV-associated miRNA was extracted with two phenol:chloroform phase separation steps, as described previously [32]. Extracted miRNA was checked via small RNA BioAnalyzer (Agilent Technologies, USA) for minimum 100pg/μl concentration in the 10–40nt band and quantified on NCounter (Nanostring Technologies, USA) with human miRNA panel v3.0 with a smaller dilution factor (1:2) for the miRNA samples to combat low EV-miRNA quantities in plasma.

miRNA analysis

All statistical analyses were performed in R (v4.2.1). EV-miRNA raw counts were first assembled using NSolver v1.0 (Nanostring) and exported. EV-miRNA counts were normalized to positive controls and log-transformed before modeling treatment. Of the 800 miRNAs in the Nanostring panel, two miRNAs (miR-450a-2-3p and miR-33a-5p) were removed from the dataset for reagent degradation among the repeated samples and 7 samples were removed from the analysis for low counts (positive normalization factor >6). Values below sample-specific detection thresholds (mean of negative controls) were assigned the machine-read values. miRNA

detected above sample-specific background (mean +1.5SD of negative controls) for either early or late pregnancy timepoints in at least 5% of samples were retained, totaling 483 miRNAs. Participants missing critical covariate data were removed, for a final retained sample of 235 participants in early pregnancy and 348 in late pregnancy.

Residential air pollution exposure assessment

Daily residential histories were constructed for each participant based on data obtained via staff-administered prospective and recall questionnaires. Residential timelines were anchored to the date of birth of the child, clearly delineated gestational periods, and captured all residential mobility. Daily concentrations of ambient air pollutants were estimated at the residential location using squared inverse distance weighting spatial interpolation for $PM_{2.5}$, PM_{10}, NO_2, and O_3 based on the dense regulatory air monitoring network data in southern California obtained from the US EPA Air Quality System [16, 75]. To capture more local impacts of traffic-related air pollution which is a significant concern in southern California [77, 78], the CALINE4 California Line Source Dispersion Model [75] was used to model the concentrations of traffic-related nitrogen oxides (traffic NO_x) to residential exposures from traffic on surrounding roads.

We calculated the prior 1-day (acute), 1-week (acute), and 8-week (subchronic) 24-h average concentrations of all ambient and traffic-related pollutants, as well as relative humidity and average temperature based on daily data and the date of the blood plasma sample used for miRNA assessment to use in health analyses. For cases in which the 8-week exposure window crossed into pre-conception time frame, the data were left-censored, using only ambient air pollution data from the gestational period.

Models and analyses

The five pollutants were evaluated in early or late pregnancy separately, and in three exposure windows (1 day, 1 week, or 8 weeks) before the date of blood sample collection. Linear mixed effects models were run in which each miRNA was modeled as the outcome with pollution levels as the exposure and the following covariates: GA at the time of sample, maternal age (standardized at 12 weeks), fetal sex (male or female), pre-pregnancy BMI (kg/m^2), parity (first child or later child), maternal education (did not complete high school, completed high school, some college), preferred language (English or Spanish), and race/ethnicity, with a random effect for chip timing to account for batch differences in quantification over the course of the cohort. Similar to previous work in this area [32, 76], covariates were selected on the following criteria: (i) known or thought to affect circulating miRNA (GA, BMI, maternal age, fetal sex, gravidity), (ii) socioeconomic status and acculturation proxy variables (preferred language, race/ethnicity, maternal education), or (iii) study variables (recruitment site, study entry time). Chip timing was used as a batch variable accounting for variation in the Nanostring quantification over time: samples were run in sets of 12 samples per chip over the course of several months. Chips from each 3–4 month period were grouped as a batch, respecting natural breaks in the laboratory core's schedule. Model results for each pollutant and window were FDR-adjusted, with a significance threshold of FDR $P < 0.05$. Beta values were scaled to the IQR range of the pollutant to better interpret the effect size [79], reflecting the percent miRNA change moving from the 25th percentile to the 75th percentile of air pollution exposure observed in this group.

Sensitivity analyses on the PM_{10} and $PM_{2.5}$ miRNA found in the main analysis were performed after exclusion of participants reporting smoking during pregnancy ($N = 10$). A permutation test was performed for $PM_{2.5}$ and PM_{10} by permuting the average PM exposures and re-running the models for all miRNA. A sensitivity analysis including three covariates for ambient weather was also performed with season of sample (winter, spring, summer, and fall), relative humidity, and average daily temperature corresponding to the window of exposure considered for $PM_{2.5}$, PM_{10}, and ozone. Additional models for $PM_{2.5}$ were conducted with an interaction term between pollutant exposure and season.

Downstream analyses of the significant miRNA used databases for assessment, including the Human MiRNA Disease Database (HMDD v4.0 [80]). Comprehensive lists of miRNA associated with lung, cardiovascular, neuroendocrine, depression, and inflammation-related diseases were assembled (List of diseases and method detail: SI Table S1 in supplementary material), by identifying each disease in the database and classifying them according to symptomology or location of disease. These collected, deduplicated lists were compared to miRNA significantly associated with air pollution after FDR adjustment. Gene targets for significant EV-miRNA associated with lung diseases or shared by $PM_{2.5}$ and PM_{10} exposure were evaluated with unidirectional miRDIP [81] v5.3, and restricted to the top score class (1%, Very High). Gene targets were assessed with PANTHER [82] v18.0 to investigate possible pathways affected by these pollution-associated EV-miRNA.

Tissue-specific expression used Diana Tools microRNATissue Expression Database (miTED) [83], which uses data from SRA (Sequence Read Archive) and TCGA (The Cancer Genome Atlas). A dataset of all available miRNAs and tissue-specific expression in healthy human lung and placenta was cross-checked with the lists of miRNAs significantly associated with $PM_{2.5}$ and PM_{10}. miRNAs were considered "expressed" in lung if counts-per-milllion values were greater than log2(median+ 1) [84].

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Author contributions

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Supplementary data

Supplementary data is available at *EnvEpig* online.

Conflict of interest: The authors have no competing interests to declare.

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Data Availability

miRNA data used in this study are available in dbGaP, under accession number: phs003194.v1.p1. Other MADRES data may also available upon request: madres@usc.edu.

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