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Fine Particulate Air Pollution and the Expression of microRNAs and Circulating Cytokines Relevant to Inflammation, Coagulation, and Vasoconstriction

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Background: MicroRNAs (miRNAs) are a key factor in epigenetic regulation of gene expression, but miRNA responses to fine particulate matter ($PM_{2.5}$) air pollution and their potential contribution to cardiovascular effects of $PM_{2.5}$ are unknown.

Objective: We explored the potential influence of $PM_{2.5}$ on the expression of selected cytokines relevant to systemic inflammation, coagulation, and vasoconstriction, and on miRNAs that may regulate their expression.

Methods: We designed a double-blind, randomized crossover study in which true and sham air purifiers were used to expose 55 healthy young adult students in Shanghai, China, to reduced or ambient levels of indoor $PM_{2.5}$ during two-week periods, and we measured the expression (mRNA and protein) of 10 serum cytokines, and miRNAs that target them, after each intervention period. We used linear mixed-effect models to estimate associations of the intervention, and time-weighted personal $PM_{2.5}$ exposures, with the cytokines, mRNA, and miRNAs; we also explored potential mediation by miRNAs.

Results: The findings were generally consistent for associations with the intervention and for associations with an interquartile range increase in time-weighted $PM_{2.5}$. Specifically, higher $PM_{2.5}$ exposure was positively associated with the expression (mRNA, protein, or both) of interleukin-1 (encoded by *IL1*), IL6, tumor necrosis factor (encoded by *TNF*), toll-like receptor 2 (encoded by *TLR2*), coagulation factor 3 (encoded by *F3*), and endothelin 1 (encoded by *EDN1*), and was negatively associated with miRNAs (miR-21-5p, miR-187-3p, miR-146a-5p, miR-1-3p, and miR-199a-5p) predicted to target mRNAs of *IL1*, *TNF*, *TLR2*, and *EDN1*.

Conclusions: Our findings require confirmation but suggest that effects of $PM_{2.5}$ on cardiovascular diseases may be related to acute effects on cytokine expression, which may be partly mediated through effects of $PM_{2.5}$ on miRNAs that regulate cytokine expression. https://doi.org/10.1289/EHP1447

Introduction

Cardiovascular disease (CVD) hospitalization and mortality attributable to fine particulate matter ($PM_{2.5}$) air pollution continues to be one of the greatest public health concerns worldwide because of ubiquitous exposure, particularly in developing countries (GBD 2015 Risk Factors Collaborators 2016; Yang et al. 2013). However, biological mechanisms that link $PM_{2.5}$ exposures with CVD events remain to be fully elucidated. It is widely accepted that PM air pollution plays a causal role in CVDs, including stroke, hypertension, heart failure, acute myocardial infarction, coronary heart disease, and atherosclerosis (Brook et al. 2010), and it has been proposed that inflammatory responses to $PM_{2.5}$ contribute to these effects (Franklin et al. 2015). Consistent with this mechanism, a recent panel study of healthy adult nonsmokers reported that serum concentrations of pro-inflammatory cytokines that may contribute to systemic inflammation, platelet activation, and endothelial dysfunction were elevated in association with ambient $PM_{2.5}$ concentrations during the previous 24 h (Pope et al. 2016).

In recent decades, the field of epigenetics has attracted increasing interest because it can serve as a bridge between environmental exposures and health-relevant events (Vrijens et al. 2015). As a main epigenetic regulatory mechanism, microRNAs (miRNAs) have been proposed to play a crucial role in regulating cellular events (Marques-Rocha et al. 2015). miRNAs comprise a large family of small noncoding RNA molecules, which function in messenger RNA (mRNA) silencing and gene expression control at the posttranscriptional level. In most cases, miRNAs lead to the inhibition of protein synthesis either by degrading mRNA transcripts or by repressing the translational process (Sevignani et al. 2006). It has been suggested that miRNA dysregulation may be involved in the development of a wide range of CVDs (Maegdefessel 2014).

Exposure to air pollutants has the potential to change the profiles of miRNAs (Jardim 2011; Rider et al. 2016). However, there is far less available evidence on miRNAs than on clinical or subclinical outcomes. In a recent systematic review, Vrijens et al. (2015) noted that although miRNA expression has been associated with air pollution exposures, findings for specific miRNAs have been inconsistent with regard to the direction of associations and among *in vitro*, *in vivo*, and human studies. Furthermore, few studies have explored the role of miRNAs as potential mediators of biological pathways linking air pollutants and CVDs (Motta et al. 2016).

We hypothesized that an acute change in $PM_{2.5}$ exposure would alter the expression of candidate miRNAs in peripheral blood. We further speculated that these miRNA alterations might mediate the expression (mRNA and protein) of cytokines relevant to systemic inflammation, coagulation, and vasoconstriction. To facilitate this investigation, we designed a randomized crossover trial with a panel of healthy young adults in which ambient $PM_{2.5}$ was naturally inhaled.

Materials and Methods

Study Design and Setting

We designed a double-blind, randomized crossover trial with two distinctly different scenarios of $PM_{2.5}$ exposure, which were

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established by the alternative use of true and sham air purifiers in Shanghai, China, where particulate air pollution is severe compared with developed countries. We recruited 60 healthy students from 17 dormitory rooms at the Jiangwan campus of Fudan University. Smokers and those living with smokers were excluded from enrollment. These rooms were randomized into two groups (8 and 9 rooms per group, respectively). From November 2015 to December 2015, one group used a true air purifier placed in the center of the room for 9 consecutive days (two weekends and five weekdays), followed by a washout period, and then used a sham air purifier under the same conditions for another 9 consecutive days (two weekends and five weekdays). The other group received the same intervention at the same time but in reverse order. A 12-d washout period was selected because we believed that it would provide sufficient time to eliminate carry-over effects from the first intervention because previous epidemiological studies have suggested that the duration of acute air-pollutant effects is <1 wk (Chen et al. 2015b; Wang et al. 2015). This period was also short enough to reduce the likelihood of confounding by time-varying factors that might influence cytokine or miRNA expression. All participants and research staff were blinded to the group assignment. To ensure blinding, the study participants were not informed that this was a crossover study; in addition, a note reading "Do not open or move" was affixed to all purifiers.

All air purifiers were identical (model KJEA200E; 3M, Shanghai, China) except for removal of the filter gauze (i.e., the high efficiency particulate air filter) in the sham group. The purifier has an airflow volume of up to $\leq 301\ m^3/h$ and an effective area of $20\ m^2$. All rooms were distributed among three adjacent dormitory buildings. The room areas were $<20\ m^2$; the rooms did not have carpets and were equipped with simple wooden furniture (beds, desks, and chairs) that had been used for ≥ 5 y. Inhabitants were asked to keep doors and windows closed during the study period so that air exchange rates would be similar across all rooms. To reduce indoor air pollution sources, all dormitory rooms were cleaned by sweeping/mopping, without the use of cleaning agents, before the onset of each intervention stage. Inhabitants were not allowed to clean or cook in the rooms during the entire study period. Therefore, similar indoor PM levels and compositions were expected for the same group at the same stage. Participants were allowed to leave their dormitories to attend classes and to go to the cafeteria but were required to stay in their rooms ≥ 12 hours per day on weekdays and ≥ 19 hours per day on weekends during each intervention period.

Our study protocol was approved by the Institutional Review Board of the School of Public Health, Fudan University, and was registered at ClinicalTrials.gov (NCT02712333). All participants provided written informed consent at enrollment.

Exposure Measurements

We calculated time-weighted personal $PM_{2.5}$ exposures for each subject over each intervention period. Specifically, we requested each subject to record their time–location information every half-hour. We used $PM_{2.5}$ measurements (per half-hour) monitored at the rooftop of the campus main building as surrogates of the exposure levels for all time spent away from the dormitory room within the campus, regardless of whether participants were inside a building. We also obtained hourly $PM_{2.5}$ data from 13 fixed-site monitoring stations across Shanghai for approximating an occasional exposure outside the campus (for example, for an internship or field work) in terms of proximity. Finally, we calculated individual exposure to $PM_{2.5}$ during each intervention period by mapping the time and location data to the corresponding $PM_{2.5}$ monitoring data. Indoor $PM_{2.5}$ was measured using MicroPEM Personal Exposure Monitors (RTI International), which can record data-logged real-time $PM_{2.5}$ concentrations in 10-s cycles. Outdoor $PM_{2.5}$ on the campus was monitored using a GRIMM EDM180 environmental dust monitor (GRIMM Aerosol Technik Ainring GmBH & Co.). To ensure the comparability of $PM_{2.5}$ data measured by the MicroPEMs and the GRIMM EDM180, parallel calibrations were performed before the start of our project.

We calculated time-weighted personal daily levels of temperature and relative humidity during each intervention period using indoor measurements obtained from a HOBO data logger (Onset Computer Corporation) in each dormitory room and ambient measurements obtained from a monitor at a fixed site of the Shanghai Meteorological Bureau.

Cytokines Test

Fasting venous peripheral blood samples (two samples from each participant) were drawn at the same time of day by a certified nurse immediately after completion of each intervention stage. The blood samples were collected at the same time of day to reduce variation due to circadian rhythms. Serum was extracted within 1 h (after centrifugation) from one of the two samples, transported directly to our laboratory, and stored at $-80^{\circ}C$. The second blood sample was prepared for RNA analysis as described below. We measured blood levels of 10 cytokines including 6 pro-inflammatory cytokines [interleukin-1 (IL-1; gene name: *IL1*), interleukin-6 (IL-6; gene name: *IL6*), tumor necrosis factor-α (TNF-α; gene name: *TNF*), toll-like receptor-2 (TLR-2; gene name: *TLR2*), CD40 ligand (CD40L; gene name: *CD40LG*), and intercellular adhesion molecule-1 (ICAM-1; gene name: *ICAM1*)], two procoagulant cytokines [tissue factor (F3; gene name: *F3*) and plasminogen activator inhibitor-1 (PAI-1; gene name: *SERPINE1*)], and two vasoconstrictors [endothelin-1 (ET-1; gene name: *EDN1*) and angiotensin converting enzyme-1 (ACE-1; gene name: *ACE*)]. These cytokines were selected because they were associated with particulate air pollution in at least two previous studies of Shanghai residents or university students performed by our group (Chen et al. 2015a, 2015b; Wang et al. 2015, 2016). We measured these biomarkers using enzyme-linked immunosorbent assays (ELISAs). All tests were performed according to the manufacturer's instructions (see Table S1 for the manufacturers and the limits of detection (LODs) of each assay kit).

RNA Analyses

The second blood sample collected after each intervention was used for RNA analyses. Total RNA was extracted using a QIAamp RNA Blood Mini kit (Qiagen) immediately (within 2 h) after the blood samples were drawn. All extractions were performed according to the manufacturer's protocols. Isolated RNA samples were stored at $-80^{\circ}C$. We measured mRNA expression for each of the 10 cytokines listed above. In addition, we selected up to two miRNAs for each cytokine based on information supporting the cytokine as a "primary target" of the miRNA(s), including "strong evidence" (reporter assay, Western blot, or qPCR) in the miRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/php/search.php#target), validation of the miRNA–mRNA relationship in the EXIQON database (https://www.exiqon.com/miRSearch), or a high score (≥ 95) for the miRNA–mRNA relationship in the miRDB database (http://www.mirdb.org/miRDB/). miRNAs related to a cytokine in more than one database were preferentially selected over other miRNAs for that cytokine. Each cytokine gene (mRNA) was related to 1–2 miRNAs, and each of the selected miRNAs was related to 1–3 cytokines (see Table S2).

Complementary DNA was synthesized using total RNA. Briefly, reverse transcription (RT) reactions were performed in a volume of 20 μL containing total RNA (250 ng), dNTPs (2.5 mM each, 2 μL), 10 × RT buffer (2 μL), random primers (1 μM, 1 μL), MMLV reverse transcriptase (Promega; 10 U/μL, 2 μL), OligodT primer (0.5 μL), RNAase inhibitor (40 U/μL, 0.3 μL) and RNase-free water (≤20 μL). The RT reaction was conducted at 16°C for 30 min, followed by 40 min at 42°C and 5 min at 85°C. Then, the products were stored at −20°C for later use. The RT mixtures were then subjected to real-time PCR reactions, which were performed using the Applied Biosystems® ViiA™ 7 Real Time PCR System (Thermo Fisher Scientific). Primers were designed with AlleleID 7 software (PREMIER Biosoft). For each sample, triplicate measurements were performed on 384-well reaction plates. The data were analyzed using ViiA7™ Software v1.2 (Thermo Fisher Scientific) with the automatic Cq setting. For both mRNA and miRNA analyses, real-time PCR was performed using TaqMan miRNA assay hydrolysis probes and Gene Expression Real-Time PCR Master Mix (both from Applied Biosystems, Thermo Fisher Scientific). A ΔCq value in a random sample was used as a calibrator, and the ΔΔCq was calculated using the ΔCq of each sample minus the calibrator. The relative quantity (RQ) of each sample was calculated using the standard relative quantification formula ($RQ = 2^{-\Delta\Delta Cq}$).

Statistical Analysis

Measured values for protein expression (cytokines), mRNAs and miRNAs were natural-log transformed for statistical analyses (Fossati et al. 2014). We also calculated the time-weighted daily levels of $PM_{2.5}$, temperature, and relative humidity during each intervention period for all subjects according to their self-administered time–location questionnaires. We used linear mixed-effect models with random-effect intercepts for each subject to estimate effects of the air purification intervention (dichotomized as "true" or "sham") and continuous $PM_{2.5}$ exposures (time-weighted averages based on indoor and outdoor exposures for each participant) on each outcome, accounting for the repeated measures for each participant (Chen et al. 2015a, 2016). All models were adjusted for age (in years), gender, body mass index (continuous), and daily average temperature and relative humidity (continuous) during each intervention period.

Furthermore, we conducted a mediation analysis to estimate the proportion of the association between $PM_{2.5}$ and cytokine expression (mRNAs or cytokines) mediated by the association between $PM_{2.5}$ and miRNA expression (Chen et al. 2016; Imai et al. 2010; Wang et al. 2016). This approach decomposes the total estimated effect of $PM_{2.5}$ on cytokine expression into an estimate of the direct effect and an estimate of the indirect effect of $PM_{2.5}$ on cytokine expression mediated by miRNA. To allow for a comparable analysis, we first standardized the exposure ($PM_{2.5}$), the mediator (miRNA), and the outcome (cytokine or mRNA) by subtracting the mean and dividing by the standard deviation (Bind et al. 2016). Estimates of the proportion of the effect of $PM_{2.5}$ on cytokine expression mediated by miRNA were generated using the "*mediation*" (version 4.4.6) package in R (version 3.1.2; R Foundation for Statistical Computing), with uncertainty estimated using the quasi-Bayesian Monte Carlo method with 1,000 simulations based on normal approximation (Imai et al. 2010). Because the assumptions required for causal interpretation were not specifically evaluated and $PM_{2.5}$ exposure was not randomized by the crossover design, we performed mediation analyses using the repeated-measures data. The mediation analyses were conducted in conjunction with the aforementioned linear mixed-effect models. We did not evaluate the possible exposure–mediator interactions because of the small sample size and the lack of sufficient variation in $PM_{2.5}$ concentrations in each interventional scenario. We report the results of mediation analyses only when there was a consistent negative direction in the pathway of $PM_{2.5}$–miRNA– mRNA or of $PM_{2.5}$–miRNA–cytokine because the negative regulation of miRNAs has been widely hypothesized.

In addition, we examined whether there were carryover effects by comparing the median responses of the two treatment orders (true-to-sham air purification vs. sham-to-true air purification) by incorporating a dummy variable of order as a fixed-effect term (i.e., "1" for the true-to-sham order, and "0" for the sham-to-true order) in the mixed-effect models with $PM_{2.5}$. The period effects were similarly assessed by adding a fixed-effect term of period (i.e., "1" for the first stage, and "0" for the second stage) in the models. Finally, an intervention × period interaction term was evaluated in the models.

All statistical analyses were performed in R (version 3.1.2; R Foundation for Statistical Computing). Effect estimates are reported as percentage differences [with 95% confidence intervals (CIs)] in outcomes associated with the air purification intervention or as interquartile range (IQR) increases in time-weighted daily average $PM_{2.5}$ concentrations. In all analyses, the Benjamini–Hochberg false discovery rate (FDR) method was used to account for multiple testing with FDRs <0.05 and <0.10 considered as "significant" and "borderline significant," respectively.

Results

Descriptive Statistics

Five participants dropped out of the study before the second intervention was completed, leaving 55 in the final analysis (28 males and 27 females), with a mean age of 20 y (range 18–22 y) and an average body mass index of 21 kg/m^2 (range 17–28). According to the self-administered questionnaires, participants spent approximately 75% of the time on average inside their dormitories during the two intervention scenarios (see Table 1), and no participants left the urban areas of Shanghai during the whole study period. Table 1 also shows 82% (on average for all rooms combined) lower $PM_{2.5}$ concentrations during true purification than during sham purification; accordingly, the time-weighted personal $PM_{2.5}$ exposure was 54% lower during true purification. $PM_{2.5}$ exposures only varied slightly among participants in the same interventional scenarios. Temperature and relative humidity were quite similar between the two interventional scenarios and did not differ much among subjects in the same interventional scenarios.

Table 2 summarizes the descriptive statistics for miRNAs, mRNAs and cytokines. There were no missing data and no data below the LODs. In general, we observed higher values for miRNAs and lower values for mRNAs and cytokines (in geometric means) in the true-purification scenario than in the sham-purification scenario.

Regression Results

As shown in Table 3, compared with the sham intervention, the true purification intervention was associated with significantly lower expression of *IL1* mRNA and protein and significantly higher expression of the single miRNA predicted for IL-1 (miR-21-5p); with significantly lower *TNF* mRNA (but not TNF-α protein) and significantly higher expression of the miRNA predicted for this cytokine (miR-187-3p); with significantly lower *TLR2* mRNA (but not TLR-2 protein), and significantly higher expression of one of the two miRNAs predicted

Table 1. Descriptive statistics of daily average environmental data in sham-purified air and true-purified air scenarios for all subjects during the two intervention scenarios.

	Sham-purified air					True-purified air				
Variables	Minimum	Mean	Median	SD	Maximum	Minimum	Mean	Median	SD	Maximum
$PM_{2.5}$ in dormitories ($\mu g/m^3$)	33.5	46.8	46.5	9.1	69.5	6.6	8.6	9.9	4.0	17.1
Time in dormitories (%)	54.4	74.9	76.4	8.3	90.5	56.1	75.5	75.7	7.0	90.0
$PM_{2.5}$ ($\mu g/m^3$)[a]	36.3	53.1	53.2	9.4	79.5	12.8	24.3	24.4	5.3	37.3
Temperature (°C)[a]	15.9	19.9	19.8	1.7	23.4	16.1	20.2	21.0	2.1	23.4
Relative humidity (%)[a]	47.3	64.3	65.1	7.4	77.9	47.3	64.6	66.0	7.8	74.1

Note: $PM_{2.5}$, particulate matter with an aerodynamic diameter <2.5 μm; SD, standard deviation.
[a]Data were calculated as time-weighted personal exposures by mapping the time and location data to the corresponding monitoring data.

for this cytokine (miR-146a-5p); and with significantly lower *EDN1* mRNA and ET-1 protein and significantly higher expression of both miRNAs predicted for this cytokine (miR-1-3p and miR-199a-5p). Finally, the intervention was associated with significantly lower *IL6* mRNA (but not IL-6 protein) but was not significantly associated with expression of the miRNA predicted for this cytokine (miR-26a-5p); similarly, the intervention was associated with significantly lower expression of F3 protein but was not associated with *F3* mRNA or with the expression of either miRNA predicted for this gene (miR-19b-3p and miR-93-5p).

Patterns of associations with an IQR increase in time-weighted $PM_{2.5}$ exposure (28 $\mu g/m^3$) were very consistent with those for the filter intervention, with inverse associations with predicted miRNAs (Table 4) and positive associations with mRNA and protein (Table 5) as exposure increased. IQR increases in personal continuous $PM_{2.5}$ exposures (time-weighted) were associated with lower estimated mean expression of 8 of the 10 miRNAs and with higher estimated mean values for all of the mRNAs and cytokines. Specifically, an IQR increase in $PM_{2.5}$ was associated with significantly higher expression of mRNA and protein for *IL1*, *IL6* (FDR 0.10 for protein), *F3*, and *EDN1*; and with significantly lower expression of the same miRNAs associated with the intervention (miR-21-5p for *IL1*, miR-1-3p for *EDN1*). Similarly, an IQR increase in $PM_{2.5}$ was associated with significantly higher *TNF* mRNA (but not TNF-α protein) and significantly lower expression of the predicted miRNA (miR-187-3p) for this cytokine; and with higher *TLR2* mRNA (FDR 0.053) (but not TLR-2 protein) and significantly lower expression of one of the two miRNAs for this cytokine (miR-146a-5p). miR-

Table 2. Descriptive statistics of miRNAs, mRNAs, and cytokines in sham-purified air and true-purified air scenarios for all subjects during the two intervention stages.

	Sham-purified air						True-purified air					
Variables	Minimum	P25	Mean[a]	P50	P75	Maximum	Minimum	P25	P50	Mean[a]	P75	Maximum
miRNAs[b]												
miR-21-5p	0.02	0.10	0.32	0.34	0.95	4.11	0.05	0.30	0.66	0.79	1.63	7.86
miR-26a-5p	0.06	0.13	0.24	0.21	0.34	2.28	0.02	0.12	0.28	0.37	0.55	8.02
miR-187-3p	0.01	0.02	0.05	0.03	0.09	0.49	0.01	0.03	0.13	0.14	0.28	7.27
miR-146a-5p	0.01	0.04	0.08	0.08	0.16	1.45	0.01	0.06	0.20	0.18	0.54	10.42
miR-19b-3p	0.04	0.08	1.61	5.59	19.64	51.85	0.02	0.09	1.21	1.74	15.76	46.72
miR-93-5p	0.06	0.14	0.33	0.38	0.72	2.89	0.04	0.11	0.27	0.33	0.59	2.13
miR-145-5p	0.01	0.02	0.08	0.07	0.25	1.14	0.01	0.03	0.14	0.10	0.36	17.4
miR-1-3p	0.01	0.02	0.07	0.06	0.23	1.88	0.01	0.06	0.22	0.19	0.49	11.70
miR-199a-5p	0.01	0.03	0.04	0.03	0.06	0.12	0.01	0.03	0.07	0.06	0.10	1.28
miR-4492	0.01	0.03	0.10	0.15	0.37	12.37	0.01	0.03	0.14	0.18	0.38	14.05
mRNAs[b]												
IL1	0.06	0.19	0.43	0.41	0.64	22.26	0.05	0.15	0.27	0.21	0.40	6.75
IL6	0.22	1.88	2.92	2.72	4.45	67.97	0.18	1.08	1.59	1.35	2.92	35.56
TNF	0.17	0.39	0.62	0.50	0.86	11.61	0.17	0.25	0.44	0.44	0.58	6.86
TLR2	0.09	0.15	0.28	0.23	0.39	6.39	0.03	0.12	0.20	0.19	0.29	2.56
CD40LG	0.07	0.52	0.83	0.75	1.17	19.05	0.12	0.41	0.64	0.70	0.87	8.98
ICAM1	0.03	0.12	0.21	0.19	0.33	5.40	0.04	0.10	0.17	0.14	0.22	3.15
F3	0.08	0.25	0.51	0.39	0.62	30.10	0.09	0.16	0.35	0.25	0.66	2.89
SERPINE1	0.28	0.54	1.12	1.12	1.67	24.62	0.15	0.61	0.96	0.91	1.41	12.56
EDN1	0.16	0.92	1.30	1.52	2.03	9.20	0.09	0.30	0.66	0.66	1.24	10.16
ACE	0.09	0.51	0.88	0.86	1.10	32.24	0.11	0.34	0.67	0.71	1.25	31.44
Cytokines												
IL-1 (ng/mL)	7	13	23	19	48	94	3	8	14	15	21	54
IL-6 (ng/mL)	1	49	88	116	203	623	1	38	59	81	173	443
TNF-α (ng/mL)	15	43	57	55	76	149	10	37	44	50	58	143
TLR-2 (ng/mL)	0.2	0.5	0.7	0.7	1.0	2.7	0.1	0.4	0.5	0.6	0.8	2.8
CD40L (pg/mL)	84	130	184	202	235	935	21	131	167	182	234	418
ICAM-1 (ng/mL)	4	5	7	7	8	27	4	5	6	6	7	13
F3 (pg/mL)	37	53	82	68	113	394	28	49	76	66	107	419
PAI-1 (ng/mL)	60	105	120	124	146	209	24	92	113	116	140	215
ET-1 (pg/mL)	2	5	8	7	12	186	2	5	7	6	10	88
ACE-1 (ng/mL)	93	151	182	187	223	428	88	134	176	180	220	341

Note: ACE-1, angiotensin converting enzyme-1; CD40L, CD40 ligand; ET-1, endothelin-1; F3, tissue factor 3; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; IL-6, interleukin-6; miRNA, microRNA; mRNA, messenger RNA; P, percentile; PAI-1, plasminogen activator inhibitor-1; TLR-2, toll-like receptor-2; TNF-α, tumor necrosis factor-α.
[a]Geometric means.
[b]Calculated using the relative quantifications formula ($2^{-\Delta\Delta Cq}$).

Table 3. Percent changes (mean and 95% confidence intervals) in miRNAs, mRNAs, and circulating cytokines comparing the true-purified air scenario with the sham-purified air scenario.

Genes	Mean	95% CI	*p*-Value	FDR
miRNAs				
miR-21-5p	133	76, 191	0.002	0.006
miR-26a-5p	15	−34, 64	0.542	0.773
miR-187-3p	143	81, 205	0.002	0.006
miR-146a-5p	111	53, 169	0.006	0.013
miR-19b-3p	6	−87, 99	0.895	0.895
miR-93-5p	7	−37, 52	0.725	0.805
miR-145-5p	58	−21, 137	0.180	0.300
miR-1-3p	148	74, 222	0.006	0.013
miR-199a-5p	76	41, 112	0.001	0.006
miR-4492	15	−48, 78	0.618	0.773
mRNAs				
IL1	−63	−102, −24	0.010	0.025
IL6	−74	−103, −46	<0.001	0.002
TNF	−38	−58, −18	0.002	0.009
TLR2	−44	−74, −14	0.013	0.027
CD40LG	−25	−51, 0	0.073	0.102
ICAM1	−26	−61, 9	0.164	0.164
F3	−61	−110, −12	0.039	0.065
SERPINE1	−22	−46, 1	0.081	0.102
EDN1	−87	−132, −42	0.004	0.013
ACE	−29	−68, 9	0.161	0.164
Cytokines				
IL-1	−56	−88, −23	0.006	0.021
IL-6	−55	−105, −4	0.064	0.128
TNF-α	−5	−26, 17	0.658	0.731
TLR-2	−2	−26, 22	0.853	0.853
CD40L	−19	−35, −2	0.039	0.097
ICAM-1	−8	−28, 13	0.466	0.583
F3	−22	−31, −13	<0.001	0.001
PAI-1	−6	−17, 6	0.339	0.485
ET-1	−21	−35, −8	0.006	0.032
ACE-1	−4	−12, 4	0.306	0.484

Note: ACE-1, angiotensin converting enzyme-1; CD40L, CD40 ligand; CI, confidence interval; ET-1, endothelin-1; F3, tissue factor 3; FDR, false discovery rate; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; IL-6, interleukin-6; miRNA, microRNA; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; TLR-2, toll-like receptor-2; TNF-α, tumor necrosis factor-α.

146a-5p was also predicted as a miRNA for *CD40LG*, which had a borderline increase in protein (FDR 0.07) but not mRNA expression (FDR 0.17) in association with $PM_{2.5}$. Unlike the finding for the filter intervention, *F3* mRNA expression was significantly increased in association with $PM_{2.5}$. However, consistent with the findings for the filter intervention, F3 protein was significantly higher in association with $PM_{2.5}$, and neither of the predicted miRNAs for F3 was associated with exposure.

Table 4. Percent changes (means and 95% CIs) in miRNAs in association with an interquartile range change (28 $\mu g/m^3$) of $PM_{2.5}$ concentrations.

miRNAs	Targeted cytokines	Percent changes	*p*-Value	FDR
miR-21-5p	IL-1, ICAM-1	−119 (−169, −70)	<0.001	0.008
miR-26a-5p	IL-6	−14 (−57, 29)	0.511	0.639
miR-187-3p	TNF-α	−51 (−91, −12)	0.028	0.051
miR-146a-5p	TLR2, CD40L, ICAM-1	−76 (126, −27)	0.012	0.030
miR-19b-3p	TLR2, F3	−83 (−178, 12.0)	0.130	0.217
miR-93-5p	ICAM-1, F3	0 (−39, 38)	0.983	0.983
miR-145-5p	PAI-1	11 (−80, 58)	0.723	0.803
miR-1-3p	ET-1	−74 (−129, −19)	0.005	0.017
miR-199a-5p	ET-1	−64 (110, −18)	0.002	0.001
miR-4492	ACE	38 (−16, 92)	0.192	0.274

Note: ACE-1, angiotensin converting enzyme-1; CD40L, CD40 ligand; CI, confidence interval; ET-1, endothelin-1; F3, tissue factor 3; FDR, false discovery rate; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; IL-6, interleukin-6; miRNA, microRNA; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 μm; TLR-2, toll-like receptor-2; TNF-α, tumor necrosis factor-α.

Estimates of the proportion of associations between $PM_{2.5}$ and mRNA or protein expression mediated by predicted miRNAs for each cytokine varied greatly, and none was significant at FDR <0.05 (Table 6). We estimated that miR-187-3p mediated 25% (95% CI: 6%, 43%), and that miR-146a-5p mediated 20% (95% CI: 2%, 38%), of the association between $PM_{2.5}$ and *TNF* mRNA (FDR 0.08 and 0.15, respectively); that miR-1-3p mediated 22% (95% CI: 5%, 39%) of the association with *EDN* mRNA (FDR = 0.08); that miR-199a-5p mediated 21% (95% CI: 5%, 38%) of the association with ET-1 protein (FDR = 0.13); and that miR-21-5p mediated 41% (95% CI: 6%, 75%) of the association with IL-1 protein (FDR = 0.13). Otherwise, estimated mediation proportions were close to the null, imprecise, or both.

In the mixed-effect models with $PM_{2.5}$ and various biomarkers, the dummy terms for order (i.e., "1" for the true-to-sham order and "0" for the sham-to-true order), period (i.e., "1" for the first stage and "0" for the second stage), and the intervention × period interaction were not significant at the 0.05 FDR level (see Table S3).

Discussion

We performed a randomized crossover study comparing miRNA and cytokine expression following an air-filtration intervention to reduce indoor $PM_{2.5}$ and a sham intervention; we also estimated associations between the outcomes and time-weighted $PM_{2.5}$ exposure estimates that accounted for both ambient and indoor $PM_{2.5}$ concentrations during the two intervention periods. The results of both analyses suggested that $PM_{2.5}$ exposure increased the expression of cytokines involved in inflammation, coagulation, and endothelial dysfunction while decreasing the expression of miRNAs that may regulate expression of these cytokines. Collectively, these results provide preliminary evidence supporting our hypothesis that short-term exposure to $PM_{2.5}$ may contribute to the pathogenesis of CVD, at least in part, by affecting the expression of circulating miRNAs.

Our findings from a randomized crossover study were consistent with those of previous epidemiological studies showing that circulating cytokines were sensitive to a short-term exposure to PM (Chen et al. 2015a, 2015b; Wang et al. 2015; Wu et al. 2012). Our results suggest that $PM_{2.5}$ might contribute to the pathophysiology of cardiovascular diseases by increasing the expression of cytokines that induce systemic inflammation (IL-1), coagulation (F3), or vasoconstriction or endothelial dysfunction (ET-1) (Franklin et al. 2015). In addition to finding evidence consistent with effects on protein transcription, we also found associations between $PM_{2.5}$ and mRNA expression for IL-1, F3, ET-1, IL-6, TNF, and TLR-2.

The mechanism by which $PM_{2.5}$ may induce cytokine expression has not been fully characterized (Marques-Rocha et al. 2015). miRNAs have emerged as important regulators in gene expression, and miRNA expression is crucial to the regulation of cellular processes. However, findings from studies of miRNA expression and air pollution exposure have been inconsistent (Jardim 2011). For example, in a recent systematic review, Vrijens et al. (2015) noted that findings for specific miRNAs from *in vitro*, *in vivo*, and human studies varied with regard to both the presence and direction of associations with air pollutants.

We utilized three online databases to identify miRNAs likely to target the cytokines of interest. However, different tools did not always predict the same miRNAs for each cytokine, reinforcing the fact that the linkages between the selected miRNAs and cytokines are not specific and are not known with certainty. Expression of eight of the 10 miRNAs measured in our study

Table 5. Percent changes (means and 95% CIs) in mRNAs and cytokines in association with an interquartile range change (28 $\mu g/m^3$) of $PM_{2.5}$ concentrations.

mRNAs				Cytokines			
Name	Percent change	*p*-Value	FDR	Name	Percent change	*p*-Value	FDR
IL1	49 (16, 82)	0.014	0.028	IL-1	41 (13, 69)	0.013	0.043
IL6	54 (27, 85)	0.002	0.010	IL-6	51 (6, 96)	0.050	0.100
TNF	28 (9, 47)	0.010	0.025	TNF-α	10 (−9, 29)	0.301	0.431
TLR2	34 (6, 62)	0.032	0.053	TLR-2	9 (−12, 31)	0.388	0.431
CD40LG	19 (−5, 42)	0.138	0.167	CD40L	17 (3, 32)	0.028	0.070
ICAM1	24 (−7, 56)	0.150	0.167	ICAM-1	3 (−15, 21)	0.741	0.741
F3	77 (37, 117)	0.004	0.020	F3	18 (9, 27)	<0.001	0.006
SERPINE1	19 (−2, 41)	0.100	0.143	PAI-1	5 (−5, 15)	0.350	0.431
EDN1	57 (23, 91)	0.008	0.025	ET-1	21 (9, 34)	0.003	0.015
ACE	6 (−29, 42)	0.713	0.713	ACE-1	3 (−4, 11)	0.359	0.431

Note: ACE-1, angiotensin converting enzyme-1; CD40L, CD40 ligand; CI, confidence interval; ET-1, endothelin-1; F3, tissue factor 3; FDR, false discovery rate; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; IL-6, interleukin-6; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 μm; TLR-2, toll-like receptor-2; TNF-α, tumor necrosis factor-α.

population was higher following the filter versus the sham intervention and lower in association with an IQR increase in time-weighted $PM_{2.5}$ exposure. Our findings of inverse associations between $PM_{2.5}$ and miR-21-5p, miR-1-3p, and miR-146a-5p were consistent with findings from the Boston Area Normative Aging Study (Fossati et al. 2014). In contrast, PM total mass was not associated with miR-21 or miR-146 in foundry workers, although some metal PM components were inversely associated with miR-146 measured by real-time PCR (Bollati et al. 2010). miR-146a expression was significantly up-regulated in postexposure versus baseline samples from the same population based on a microarray analysis of 847 human miRNAs (Motta et al. 2013). In addition, miR-21 expression was higher following exposure to diesel exhaust (vs. filtered air) in a controlled-exposure crossover study of 14 asthma patients (Yamamoto et al. 2013). Inconsistent findings among studies of PM and miRNA expression may reflect differences in PM characteristics, study participants, or study designs, although random error and noncausal explanations must also be considered. Nevertheless, although this field is still in its preliminary stages, our findings and those of previous investigators merit further investigation and confirmation.

Altered miRNA expression may trigger a cascade of cytokines involved in the complex biological processes that contribute to cardiovascular diseases (Motta et al. 2013), but our understanding of the role of miRNAs in complex molecular networks is limited. Consistent with our *a priori* hypothesis, we found preliminary evidence supporting positive mediation of the effects of $PM_{2.5}$ at both the transcriptional (mRNA) and translational (protein expression) levels by miRNAs. However, the validity of the estimates from the mediation analysis depends on several unverifiable assumptions, including the absence of uncontrolled confounding of associations between the exposure and the outcomes, between the exposure and the mediator, and between the mediator and the outcomes. In particular, we note that although the filter intervention was randomized, the time-weighted $PM_{2.5}$ exposures used as the basis of the mediation analysis were not. In addition, the analysis was further complicated by the fact that each miRNA can target multiple mRNAs/proteins, and a single mRNA/protein is often the target of multiple miRNAs (Motta et al. 2013).

Our study had several strengths. First, we enrolled healthy students living in university dormitories to reduce potential confounding from indoor air pollution, smoking, alcohol drinking, medication use, dietary structure, time–activity patterns, sociodemographic characteristics, and socioeconomic status (Chen et al. 2015a, 2016). Second, this interventional crossover design with true and sham air purifiers created large self-contrasts in $PM_{2.5}$ exposures that are common in developing countries, making it easier to capture potential molecular changes in response to $PM_{2.5}$ (Chen et al. 2016). Third, circulating cytokines, mRNAs, and miRNAs were evaluated simultaneously in our analysis, allowing for a preliminary assessment of the potential mediation or modulation of miRNAs in the cardiovascular effects of $PM_{2.5}$.

Table 6. The mediation proportion (%, means and 95% confidence intervals) by microRNAs in associations of $PM_{2.5}$ with mRNAs and circulating cytokines.

	mRNAs				Cytokines			
miRNAs	Name	Percent	*p*-Value	FDR	Name	Percent	*p*-Value	FDR
miR-21-5p	*IL1*	8.41 (17.35, 34.17)	0.51	0.85	IL-1	40.50 (6.43, 74.57)	0.02	0.13
miR-26a-5p	*IL6*	4.27 (−7.52, 16.06)	0.47	0.85	IL-6	2.19 (−7.57, 11.95)	0.65	0.99
miR-187-3p	*TNF*	24.65 (5.92, 43.38)	0.01	0.08	TNF-α	2.57 (−8.88. 14.02)	0.66	0.99
miR-146a-5p	*TNF*	20.01 (2.26, 37.76)	0.03	0.15	TNF-α	10.04 (−19.33, 39.41)	0.50	0.99
miR-146a-5p	*TLR2*	12.04 (−53.51, 77.59)	0.71	0.99	TLR-2	8.44 (−16.25, 33.13)	0.49	0.99
miR-19b-3p	*TLR2*	—	—	—	TLR-2	5.60 (−6.33, 17.53)	0.36	0.99
miR-146a-5p	*CD40LG*	28.47 (−13.17, 70.11)	0.19	0.71	CD40L	—	—	—
miR-21-5p	*ICAM1*	13.74 (−73.13, 100.61)	0.76	0.99	ICAM-1	0.10 (−6.43, 6.63)	0.99	0.99
miR-93-5p	*ICAM1*	0.50 (−4.94, 5.94)	0.86	0.99	ICAM-1	0.30 (−7.05, 7.65)	0.95	0.99
miR-146a-5p	*ICAM1*	5.32 (−10.24, 20.88)	0.49	0.85	ICAM-1	4.39 (−4.96, 13.74)	0.36	0.99
miR-19b-3p	*F3*	5.47 (−4.46, 15.40)	0.28	0.84	F3	—	—	—
miR-93-5p	*F3*	0.21 (−5.28, 5.70)	0.94	0.99	F3	0.75 (−6.60, 8.10)	0.84	0.99
miR-145-5p	*SERPINE1*	1.76 (−17.40, 20.92)	0.85	0.99	PAI-1	0.29 (−4.08, 4.66)	0.91	0.99
miR-1-3p	*EDN1*	22.39 (5.38, 39.40)	0.01	0.08	ET-1	2.92 (−19.97, 25.81)	0.79	0.99
miR-199a-5p	*EDN1*	4.52 (−7.96, 17.00)	0.47	0.85	ET-1	21.40 (5.14, 37.66)	0.01	0.13
miR-4492	*ACE*	0.13 (−8.36, 8.62)	0.99	0.99	ACE-1	—	—	—

Note: —, invalid results of mediation analyses when there was a positive direction in the pathway of $PM_{2.5}$-miRNA − mRNA or of $PM_{2.5}$-miRNA-cytokine according to our hypothesis; ACE-1, angiotensin converting enzyme-1; CD40L, CD40 ligand; CI, confidence interval; ET-1, endothelin-1; F3, tissue factor 3; FDR, false discovery rate; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; IL-6, interleukin-6; miRNA, microRNA; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 μm; TLR-2, toll-like receptor-2; TNF-α, tumor necrosis factor-α.

Some limitations should also be noted. First, power and precision were somewhat limited because of the small sample size. Second, studying young, healthy subjects and special exposure scenarios reduced the potential for uncontrolled confounding but may limit generalizability to other populations and contexts. Third, we cannot assume that estimates from our mediation analyses represent causal mediation of an effect of $PM_{2.5}$ by miRNAs because it is not possible to confirm underlying assumptions required for this to be true, including the absence of some uncontrolled confounding of associations between $PM_{2.5}$ and miRNAs, between miRNAs and the outcomes, and between $PM_{2.5}$ and the outcomes (Valente et al. 2017). Fourth, experimental control was limited because students left their dormitory rooms to attend classes and to go to the cafeteria, and the intervention was therefore not the sole influence on $PM_{2.5}$ exposures during the sham or the filter intervention periods. We also estimated the effects of time-weighted $PM_{2.5}$ exposures that accounted for ambient $PM_{2.5}$ concentrations and time–activity patterns, but these exposures were not randomized, and some degree of exposure misclassification and confounding was likely. Fifth, we did not have data on the proportions of different leukocyte subpopulations in the participants' blood samples, which may influence both miRNA and cytokine expression (Fossati et al. 2014) and may vary over short periods of time. Therefore, we cannot rule out the possibility of uncontrolled bias from this source of variation. Finally, we measured $PM_{2.5}$ only and cannot rule out the possibility that larger or smaller PM might have been responsible for the estimated associations with $PM_{2.5}$.

Conclusion

In summary, we performed a randomized crossover trial of a sham versus true air filtration intervention and estimated associations with time-weighted $PM_{2.5}$ exposures (indoor and outdoor exposures) in healthy university students in Shanghai, China. We identified several miRNAs that were inversely associated with $PM_{2.5}$ exposure and found that the expression of several cytokines (mRNA and/or protein) predicted to be targets of the same miRNAs was positively associated with $PM_{2.5}$ exposure. These findings need to be confirmed in other populations and experimental models, but they provide preliminary evidence that acute responses and pathophysiologic mechanisms that may link $PM_{2.5}$ exposure with cardiovascular disease, including systemic inflammation and endothelial dysfunction, may be partly mediated by effects of $PM_{2.5}$ on miRNA expression.

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