



Urine metabolites associated with cardiovascular effects from exposure of size-fractioned particulate matter in a subway environment: A randomized crossover study

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ABSTRACT

Background: Ambient particulate matter (PM) is closely associated with morbidity and mortality from cardiovascular disease. Urine metabolites can be used as a non-invasive means to explore biological mechanisms for such associations, yet has not been performed in relation to different sizes of PM. In this randomized crossover study, we used metabolomics approach to explore the urine biomarkers linked with cardiovascular effects after PM exposure in a subway environment.

Methods and results: Thirty-nine subjects were exposed to PM for 4 h in subway system, with either a respirator intervention phase (RIP) with facemask and no intervention phase (NIP) in random order with a 2-week washout period. Electrocardiogram (ECG) parameters and ambulatory blood pressure (BP) were monitored during the whole riding period and urine samples were collected for metabolomics analysis. After exposure to PM for 4 h in subway system, 4 urine metabolites in male and 7 urine metabolites in female were screened out by UPLC/Q-TOF MS/MS-based metabolomics approach. Cardiovascular parameters (HRV and HR) predominantly decreased in response to all size-fractions of PM and were more sensitive in response to different size-fractioned PM in males than females. Besides LF/HF, most of the HRV indices decrease induced by the increase of all size-fractioned PM while PM_{1.0} was found as the most influential one on indicators of cardiovascular effects and urine metabolites both genders. Prolyl-arginine and 8-OHdG were found to have opposing role regards to HRV and HR in male.

Conclusion: Our data indicated that short-term exposure to PM in a subway environment may increase the risk of cardiovascular disease as well as affect urine metabolites in a size dependent manner (besides PM_{0.5}), and male were more prone to trigger the cardiovascular events than female after exposure to PM; whereas wearing facemask could effectively reduce the adverse effects caused by PM.

1. Introduction

Cardiovascular disease is the leading cause of total years of life lost (YLLs) globally (GBD CoD Collaborators, 2017). A growing number of epidemiological evidences show that cardiovascular disease is closely linked with ambient particulate matter (PM). The well-known scientific statements of American Heart Association (AHA) states that an elevation of 10 µg/m³ particulate matter with aerodynamic diameter < 2.5 nm (PM_{2.5}) is associated with 8–18% increases in risk for

cardiovascular mortality (Pope 3rd et al., 2004). PM_{2.5} has been recognized as an environmental risk factor and severe public issue to human health. It is estimated that the attributable burden contributed by ambient particulate matter pollution estimation is ~12% of the global burden of disease (GBD, 2017). Levels of air pollutants in underground subway stations have attracted attention in recent years, since these subway systems with rather confined space will unavoidable accumulate and increase the concentrations of air pollutants (Moreno and de Miguel, 2018). Evidence shows that the PM_{2.5} concentrations in

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subways are much higher than levels above ground in cities, such as London, New York, Berlin, Beijing and Barcelona (Martins et al., 2016; Vilcassim et al., 2014). Currently, subway is almost the first transport choice for both commuters and travelers in urban areas to reach the destination without road congestion or time wasting (Moreno et al., 2017). Therefore, the air quality conditions in the subway systems may have a significant health impact on a large population.

Largely, studies of air pollution in subways have concentrated on the monitoring and characterization of airborne particle concentrations or sources in subway environment, with few studies addressing the effects of PM exposure on human health (Grana et al., 2017; Minguillon et al., 2018). Of the limited studies available, it has been shown that asthmatics are more sensitive to subway PM than healthy individuals, with increase in CD25-expressing of CD4 cells and alterations in oxylipin profile in bronchoalveolar lavage fluid (Klepczynska-Nystrom et al., 2012). Healthy individuals showed no inflammatory response in the lower airways, but had statistically significant increase in fibrinogen and regulatory T-cells expressing CD4/CD25/FOXP3 in the peripheral blood (Klepczynska Nystrom et al., 2010). In contrast, Bigert et al. found no significant change in short-term respiratory effects (airway inflammation or lung function) of subway employees before and after a working day (Bigert et al., 2011). Our previous intervention study found that short-term wearing of a facemask/headphone could reduce the cardiovascular risk and improve the autonomic nervous function of healthy individuals exposed to PM in Beijing subway (Yang et al., 2018). But what kinds of biomarkers were mostly changed in human subjects exposed to subway environment and which biomarkers could be closely linked with PM-induced cardiovascular effects were still largely unknown. In the present study, we investigate the urine metabolites between exposure to PM in subway and intervention with facemask, and whether these potential biomarkers related to PM-induced cardiovascular effects.

Metabolomics can be used as a high-throughput tool for biomarker discovery to identify perturbations of small-molecules (< 1 kDa) in biofluids, cells or tissues (Nicholson and Lindon, 2008). Metabolomics provides 'functional' information which is helpful for understanding the systems-level effects of metabolites and provide insight into the mechanisms that underlie various physiological conditions of diseases (Johnson et al., 2016). The main methodologies that are used for metabolite recovery and identification are untargeted (global) and targeted mass spectrometry-based metabolomics. Currently, the metabolomics approach as an emerging method has been applied in air pollution research.

Menni et al. found that 21 metabolites were associated with lung function, 8 of which were also associated with PM_{2.5} and PM₁₀ long-term exposure (Menni et al., 2015). A targeted metabolomics-based study revealed that short-term exposure to ambient PM_{2.5} and ozone was associated with alterations in plasma acylcarnitines and amino acids in a cohort of cardiac catheterization patients (Breitner et al., 2016). In a recent study, Li et al. reported that higher exposed to PM increased cortisol, cortisone, epinephrine and norepinephrine, suggesting that PM exposure is closely linked with the stress hormone levels of human subjects (Li et al., 2017). However, it is still unknown if there is a size-dependent relationship between PM and metabolites, and which kinds of metabolites could effectively and accurately reflect the cardiovascular changes linked to PM.

Based on above evidences, we conducted a randomized crossover study in healthy subjects exploring the urine metabolites changes associated with short-term exposure of size-fractioned PM in Beijing subway system with or without respirator intervention. The study used a UPLC/Q-TOF MS/MS based metabolomics approach to explore the associations of metabolism and cardiovascular effects to subway PM and provide scientific data to clarify the health influencing mechanism of particulate matter, which could further provide an effective and noninvasive way to predict the potential air pollution-related cardiovascular diseases by detecting the urine biomarkers.

2. Materials and methods

2.1. Study design and participants

This randomized crossover study was performed on 39 college students traveling in the underground subway in Beijing within the period of March to May 2017. Demographic information such as age, sex, height, weight, and disease history were collected at enrollment. All participants were healthy young adults, non-smokers, and did not take any medicine. Written informed consent was obtained from each subject before enrollment. The study was approved by Review Board of Peking University Health Science Center (RIB00001052-16066).

Respirator intervention phase (RIP) and no intervention phase (NIP) were allocated for each subject in random order with two-week washout period. Subjects travelled in the underground subway from 09:00 to 13:00 each study period. Electrocardiogram (ECG) parameters and ambulatory blood pressure (BP) were monitored during throughout the riding period. Subjects accepted dietary guidance and were instructed to avoid taking vigorous exercise the day before the study. In respirator intervention phase, subjects were instructed to ensure a perfect fit with their face to minimize the penetration of PM through the gaps between the face and the respirator. The respirator package was a 3 M respirator (9002 V) connected to a pump with an efficient filter. Full study procedures were described elsewhere in detail (Yang et al., 2018).

2.2. PM exposure measurements

The concentrations of size-fractioned PM, including PM_{0.5}, PM_{1.0}, PM_{2.5}, PM_{5.0} and PM₁₀, were measured using a real-time particulate counter (Model Handheld PC3016; GrayWolf Inc., USA). We estimated the filtration rates of respirator using two instruments, which simultaneously measured PM concentrations inside and outside the respirator in order to calculate the real PM exposure parameter (inside the respirator mask) in RIP. The air inside the respirator is connected to the instrument through a sampling pipe for air pollutant monitoring which was guaranteed the air-tightness. Considering that the respirator used in our study might have different filtration rates for PM with different size, the calculation formula of the filtration rates is: Filtration rate for PM_x = 1 - PM_x average concentration inside the respirator / PM_x average concentration outside the respirator * 100%. Noise levels were measured using a portable noise meter (Model ASV5910; Hangzhouaihua Inc., Hangzhou, China). Real-time temperature (Temp) and relative humidity (RH) were recorded by a Temp/RH meter in 1-min intervals (Model WSZY-1B; Tianjianhuayi Inc., Beijing, China). All exposure data (size-fractioned PM, Temp and RH) were aggregated as 5-min averages to match with 5-min HRV measurements.

2.3. Health measurements and sample collection

Each participant wore a 12-channel Holter recorder (model MGY-H12; DM Software Inc., USA) to collect ECG parameters throughout the study period. The ECG digital records were processed by trained technicians to extract Heart Rate Variability (HRV) and Heart Rate (HR) data using the computer-based software (Holter System, version 12.net; DM Software Inc., USA). Four time-domain parameters were calculated: standard deviation of all NN intervals (SDNN); standard deviation of the averages of NN intervals in all 5-min segments of the entire recording (SDANN); root mean square of successive differences between adjacent NN intervals (rMSSD); and percentage of number of NN interval with difference ≥ 50 ms (pNN50). Four frequency-domain parameters were also measured by Welch's averaged periodogram of the NN intervals: total power (TP), the area under the spectral curve between 0.01 and 0.4 Hz; VLF, power in the very low frequency band (0.01–0.04 Hz); LF, power in the low frequency band (0.04–0.15 Hz); HF, power in the high frequency band (0.15–0.4 Hz);

and LF/HF ratio, low to high frequency power ratio averaged. All parameters were calculated in 5-min segments.

Ambulatory BP was measured by an ambulatory BP monitor (Mobile-O-GEAPH NG Vers.20; Hypertension Management Software Inc. Germany) placed over the left brachial artery. BP was measured every 15 min during the study period.

The urine samples (10 mL) from each participant were collected into sterilized tubes at the end of each study period. All the participants were required to empty the bladder in the morning before taking the subway. All samples were transported at 4 °C within 2 h and stored at −80 °C until analysis. We conducted all examinations and collected samples at the same time of the day to minimize potential influence from physiological changes attributable to circadian rhythm.

2.4. Metabolomics analysis

Prior to analysis, urine samples were thawed at 4 °C, diluted 1:3 (v/v) with water, vortex-mixed for 60 s, and then centrifuged at 15000 rpm for 15 min. The supernatants were transferred to vial inserts and placed in autosampler vials.

A 2 µL sample solution was injected into an Acquity UPLC HSS T3 column (100 × 2.1 mm; id. 1.7 µm; Waters) at 35 °C using the Waters Acquity UPLC system (Waters, Milford, MA). The flow rate of the mobile phase was 0.35 mL/min. Analytes were eluted from the column under a gradient (solvent A, 0.1% formic acid in water; solvent B, acetonitrile). The elution gradient was as follows: 2% B for 0.5 min, 2–20% B over 0.5–7.0 min, 20–35% B over 7.0–9.0 min; 35–70% B over 9.0–13.0 min; 70–98% B over 13.0–14 min; 98% B for 4.0 min; returned to 2% B for 0.5 min; 2% B for 2.5 min. Acetonitrile was run every fifth sample as a blank solution. Urine samples in two analysis batches were injected alternately between three RIP and three NIP.

Mass spectrometry was performed using a Waters Q-TOF SYNAPT G2-Si mass spectrometer (Waters, Manchester, UK) with electrospray ionization (ESI) in the positive and negative modes. The parameters were as follows: data was collected from m/z 50 to 1050; the desolvation gas (nitrogen) flow was set at 800 L/h; cone gas (nitrogen) flow was set at 50 L/h; source temperature, 120 °C; desolvation temperature, 450 °C; capillary voltage, 1.0 kV; scan time was set for 0.6 s; MS^E analysis was performed with two scan functions (the low collision energy was off, and the high collision energy was set at 20 to 60 V) for simultaneous acquisition of precursor ion information and MS2 data at low and high collision energy in one analytical run. A lock mass of leucine enkephalin ($[M + H]^+ = 556.2771$; $[M + H]^- = 554.2615$) was applied via a lock spray interface. Lock spray frequency was set at 0.6 s, and lock mass data were averaged over 10 scans for correction.

To verify the reproducibility of the data, we prepared a pooled quality-control (QC) sample by mixing equal volumes of urine samples from participants and analyzed every 5th samples throughout the analytical run. The reproducibility of the method was determined with principal component analysis (PCA) in SIMCA-P 13.0 software (Umetrics). PCA performed on QC and all the other samples revealed that QC samples were clustered in the PCA scores plot (Fig. S1). These results collectively indicated good repeatability, reliability and stability of this method for metabolite analysis.

2.5. Data processing and statistical analysis

Data processing was conducted using Progenesis QI (Waters, Milford, MA, USA). After peak picking and alignment, data was normalized to MS 'total useful signal' (MSTUS) using the total intensity of peaks. MSTUS using the total intensity of peaks that are present in all samples under study as the normalization factor, and it is thus more universally applicable and widely used in several applications in recent years (Gagnebin et al., 2017; Sussulini, 2017). MSTUS normalization could provide comparable or better performance than other common approaches in the non-targeted urinary metabolome analysis (Di Guida

et al., 2016) Progenesis QI with built-in Metascope search engine enables to search ID of compounds using up to 5 different criteria including exact mass, MS/MS fragments, isotope similarity, retention time and collisional cross-section by searching against famous HMDB (<http://www.hmdb.ca/metabolites>), METLIN (<http://metlin.scripps.edu/index.php>), ChempSpider (<http://www.chemspider.com>) database and 'in silico' fragmentation database. The metabolite candidate was identified according to its ID score which was computed from the ID criteria mentioned above. Endogenous metabolites with a score value > 30 were selected, and the score value of the compound is the higher the better in identification. The chemical structure and corresponding identification data of selected metabolites are shown in Fig. S2 and Table S1. The significance changes of ions were analyzed by paired Student's *t*-tests (NIP vs RIP), with $p < 0.05$ deemed to be statistically significant. Multiple comparisons for this data set were also accounted with false discovery rate method, and each false discovery rate was estimated using *q* values, with a $q < 0.10$ considered significant. To identify potential biomarkers associated with respirator wearing, we selected metabolites with $p < 0.05$, $q < 0.10$ and fold changes of peak area > 1.5 or < 0.67.

Paired Student's *t*-test was used to investigate the difference of PM exposure and health parameters between NIP and RIP. Before the statistical analysis, HRV and HR data were log10-transformed to improve the normal distribution. The threshold effect was evaluated by R 3.5.0 software and results were shown in Fig. S3–S5. Then we applied linear mixed-effect models with a random intercept for each subject to estimate the associations for PM exposure parameter inside the mask (data in RIP were adjusted according to exposure measurements and filtration rates), health measurements and peak intensity for each significantly changed metabolites. Data was adjusted for age, BMI, the mean noise, temperature, relative humidity and number of the measured days (encoding method: measurement date - start date of this study). The correlation and collinear test results between covariates are shown in Table S2–S3. Each HRV parameter effects were presented as the mean percentage change, obtained by $(10^{\beta-1}) \times 100\%$, with 95% confidence intervals (CI), $(10^{\beta \pm 1.96 \times SE-1}) \times 100\%$, where β and SE were the regression coefficient and its standard error, of the other modes of interventions compared with the reference. Metabolites changes were expressed as changes in absolute values and 95% CIs. All statistical analyses were performed using the 'nlme' package for R 3.5.0 software, and statistically significant differences were considered as $p < 0.05$.

3. Results

3.1. Baseline characteristics, exposure and health measurements

Data was obtained from 39 participants, including 21 males and 18 females. Average age and BMI were 21.4 ± 0.0 years and 22.1 ± 2.3 kg/m² for males and 20.9 ± 0.5 years and for 21.1 ± 0.8 kg/m² females (Supplemental Table S4). Detailed exposure data are shown in Table S5. The mean levels of personal PM_{0.5}, PM_{1.0}, PM_{2.5}, PM_{5.0}, PM₁₀ and TPM exposure were 12.15 µg/m³, 34.71 µg/m³, 86.77 µg/m³, 199.94 µg/m³, 232.36 µg/m³, 257.93 µg/m³ in NIP and 11.43 µg/m³, 32.06 µg/m³, 76.38 µg/m³, 177.23 µg/m³, 203.03 µg/m³, 226.94 µg/m³ in RIP, respectively. No significant differences of exposure data in PM, noise, temperature and relative humidity levels were observed between NIP and RIP. The filtration of respirator for PM_{0.5}, PM_{1.0}, PM_{2.5}, PM_{5.0}, PM₁₀ and TPM were 97.42%, 97.60%, 98.01%, 98.50%, 98.74% and 98.76%, providing real exposure levels of 0.01 µg/m³, 0.02 µg/m³, 0.03 µg/m³, 0.04 µg/m³, 0.03 µg/m³ and 0.03 µg/m³ respectively.

The distribution of 5-min HRV, HR and BP in NIP and RIP for males and females were presented in Table 1. Males had significantly higher HF power values in RIP compared with NIP ($p < 0.05$), while LF/HF was lower in NIP ($p < 0.05$). As shown in Table S6, the coefficient of PM and gender's interaction in linear mixed-effects regression models.

Table 1

Comparisons of cardiovascular outcomes between participants wearing with mask and without mask during the study periods.

Parameters	Male		Female	
	NIP	RIP	NIP	RIP
HRV				
TP (ms ²)	4611.1 (2111.3)	5445.5 (2782.7)	3858.8 (168.3)	3506.5 (179.6)
VLF power (ms ²)	2941.3 (1374.1)	3456.1 (1948.0)	2344.0 (174.5)	2055.9 (326.1)
LF power (ms ²)	1222.1 (623.5)	1322.8 (546.7)	931.2 (26.2)	881.6 (10.5)
HF power (ms ²)	421.7 (252.7)	635.4 (506.3)	539.8 (30.4)	528.0 (133.8)
LF/HF	4.4 (2.0)	3.4 (1.7) *	2.7 (0.7)	2.4 (1.8)
SDNN (ms)	69.1 (17.7)	74.7 (20.4)	63.8 (1.5)	61.8 (0.3)
SDANN (ms)	26.1 (2.3)	26.6 (2.3)	27.2 (0.0)	26.4 (0.9)
rMSSD (ms)	36.5 (11.1)	43.0 (17.9)	42.0 (0.7)	40.6 (3.9)
pNN50 (%)	17.2 (9.7)	22.2 (14.1)	21.4 (0.8)	20.8 (3.7)
Heart rate (bpm)	78.4 (8.6)	76.7 (8.9)	77.6 (1.9)	77.7 (2.5)
Blood pressure				
SBP (mm Hg)	122.4 (9.8)	121.0 (9.6)	111.9 (2.8)	111.2 (4.0)
DBP (mm Hg)	77.8 (7.4)	77.0 (6.7)	71.0 (2.8)	71.7 (5.8)
PP (mm Hg)	44.5 (7.2)	42.7 (7.2)	40.8 (5.6)	40.8 (3.1)

Values presented as mean (SD); **p* < 0.05 from paired student's *t*-test. The tests for HRV were based on log10-transformed data; Abbreviation: LF, low frequency; HF, high frequency; SDNN, the standard deviation of the normal-to-normal interval; SDANN, standard deviation of the averages of NN intervals in all 5-min segments of the entire recording. rMSSD, the root mean square of the successive differences; pNN50, percentage of normal RR intervals with duration > 50 msec different from the previous normal RR interval; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.

3.2. Health measurements in relation to PM exposure

Fig. 1 shows the estimated percent changes of HRV indices and HR per IQR increase in size-fractioned PM. The most affected HRV parameters in males were HF, SDNN, rMSSD and LF/HF, with all showing statistically significant alterations with increasing PM levels for of all size-fractions. LF/HF is the only index of HRV that changed in females in response to PM. The largest alterations in health parameters were found for PM_{1.0} in both male and female groups. In males, an IQR (36.21 µg/m³) increase of PM_{1.0} was significantly associated with a 16.68% (95% CI: −26.73%, −5.24%) decline in TP (*p* = 0.023), a 31.79% (95% CI: −44.64%, −15.97%) decline in HF (*p* = 0.003), a 10.95% (95% CI: −17.87%, −3.44%) decline in SDNN (*p* = 0.016), a 15.26% (95% CI: −26.46%, −2.35%) decline in rMSSD (*p* = 0.041), a 28.14% (95% CI: −46.20%, −4.02%) decline in pNN50 (*p* = 0.044), a 30.46% (95% CI: 9.87%, 54.80%) increase in LF/HF (*p* = 0.010), and a 5.47% (95% CI: 1.77%, 9.31%) increase in HR (*p* = 0.014), respectively. In females, an IQR (29.86 µg/m³) increase of PM_{1.0} average concentration was significantly associated with a 21.09% (95% CI: 1.81%, 44.03%) increase in LF/HF (*p* = 0.049). Other sizes of PM showed a similar direction of change as PM_{1.0} with the smaller the particle size, the greater effect on HRV and HR.

3.3. Urine metabolites identification

The detected ion fragmentations were 8629 for ESI⁺ mode and 8246 for ESI[−] mode. The fragmentations that met the criterion of fold change > 1.5 or < 0.67, *p* < 0.05 and *q* > 0.10 were selected and confirmed by available databases, which showed significant differences between NIP and RIP (Table 2). Finally, compare to NIP, prolyl-arginine, α-ketoglutaric acid were significantly increased; molybdopterin precursor Z and 8-hydroxy-deoxyguanosine (8-OHdG) were markedly decreased in RIP in males. The following metabolites were altered in females in RIP; including increased: 5-hydroxylysine, *N*-

acetylglutamine, homovanillic acid and L-citrulline; decreased: γ-glutamyl ornithine, 2-oxovaleric acid and 5'-phosphoribosyl-*N*-formylglycinamide.

3.4. Metabolites in relation to PM exposure

Fig. 2 shows the estimated percent changes (peak area) of metabolites per IQR increases in size-fractioned PM. Molybdopterin precursor Z and 8-OHdG in males and *N*-acetylglutamine and homovanillic acid in females showed a strong correlation with PM. The greatest increase in 8-OHdG and homovanillic acid were found to be induced by PM_{1.0}, while molybdopterin precursor Z and *N*-acetylglutamine were most greatly affected by PM_{2.5}. An IQR increase of PM_{1.0} was associated with 132.69 (95% CI: 48.87, 216.51) increase in peak area of 8-OHdG and 428.85 (117.91, 739.79) reduction in homovanillic acid. An IQR increase of PM_{2.5} was associated with an increase of 466.17 (95% CI: 163.72, 768.62) peak area in molybdopterin precursor Z and a reduction of 2.62 (95% CI: −4.95, −0.29) peak area in *N*-acetylglutamine. Similar to the relationship between particle size and health measurements, the smaller in the particle size (besides PM_{0.5}), the greater effects on the changes in metabolites.

3.5. Metabolites in relation to HRV indices and HR

Linear mixed-effect models with a random intercept for each subject were further used to estimate the associations between selected metabolites and health measurements (Fig. 3). Prolyl-arginine and 8-OHdG in males were significantly elevated where there were increases in most HRV indices (except for LF/HF) and decrease in LF/HF and HR. 8-OHdG showed the greatest association with HRV and HR (including TP, LF, HF, SDANN, SDNN, rMSSD, pNN50, LF/HF and HR) where as IQR increase were associated with changes from −132.71 to 137.49 in peak area. SDNN and SDANN represents the overall heart rate variability, that is, the total regulation of autonomic nerves to heart rate and heart rhythm. rMSSD and pNN50 mainly represent the rapid change of HRV, which is a sensitive indicator of vagal tone. TP is the total variability of the signal, from the sum of VLF, LF, and HF; LF is affected by both sympathetic and parasympathetic nerves while HF is mediated by the vagus nerve, and LF/HF indicates the equilibrium state of sympathetic-vagal tension. The decrease of TP, LF, HF, SDANN, SDNN, rMSSD, pNN50 and the increase of LF/HF suggest the deregulation of the vagus nerve, a phenomenon which could lead to unstable myocardial electrical activity, thereby increasing the risk of re-entry tachycardia or ventricular fibrillation. The experimental design flowchart of a randomized crossover study was presented in Fig. 4.

4. Discussion

Increasingly, more city center people are choosing to commute by subway instead of any other modes of transport due to its convenience and efficiency, despite concerns over the air quality of subway systems. A growing body of evidence finds substantially higher particle concentrations in subway environments, with the potential to have adverse biological impact on living organisms (Jung et al., 2012). In this study, we used a metabolomics analysis of urine biomarkers as a noninvasive means to explore the biological mechanisms by which size-fractionated subway air particles could lead to cardiovascular effects. The approach may have practical benefits to quickly and easily identify special metabolites that could predict the susceptible population in taking subway from suffering cardiovascular events.

In this randomized crossover intervention study, we found that the urine metabolite 8-OHdG was markedly increased in NIP of healthy male volunteers, indicating that short-term exposure to PM could lead to measurable oxidative damage to DNA (Table 2). Reactive oxygen species (ROS) and oxidative stress is one of the most widely accepted mechanisms by which PM can have detrimental health outcomes (Pardo

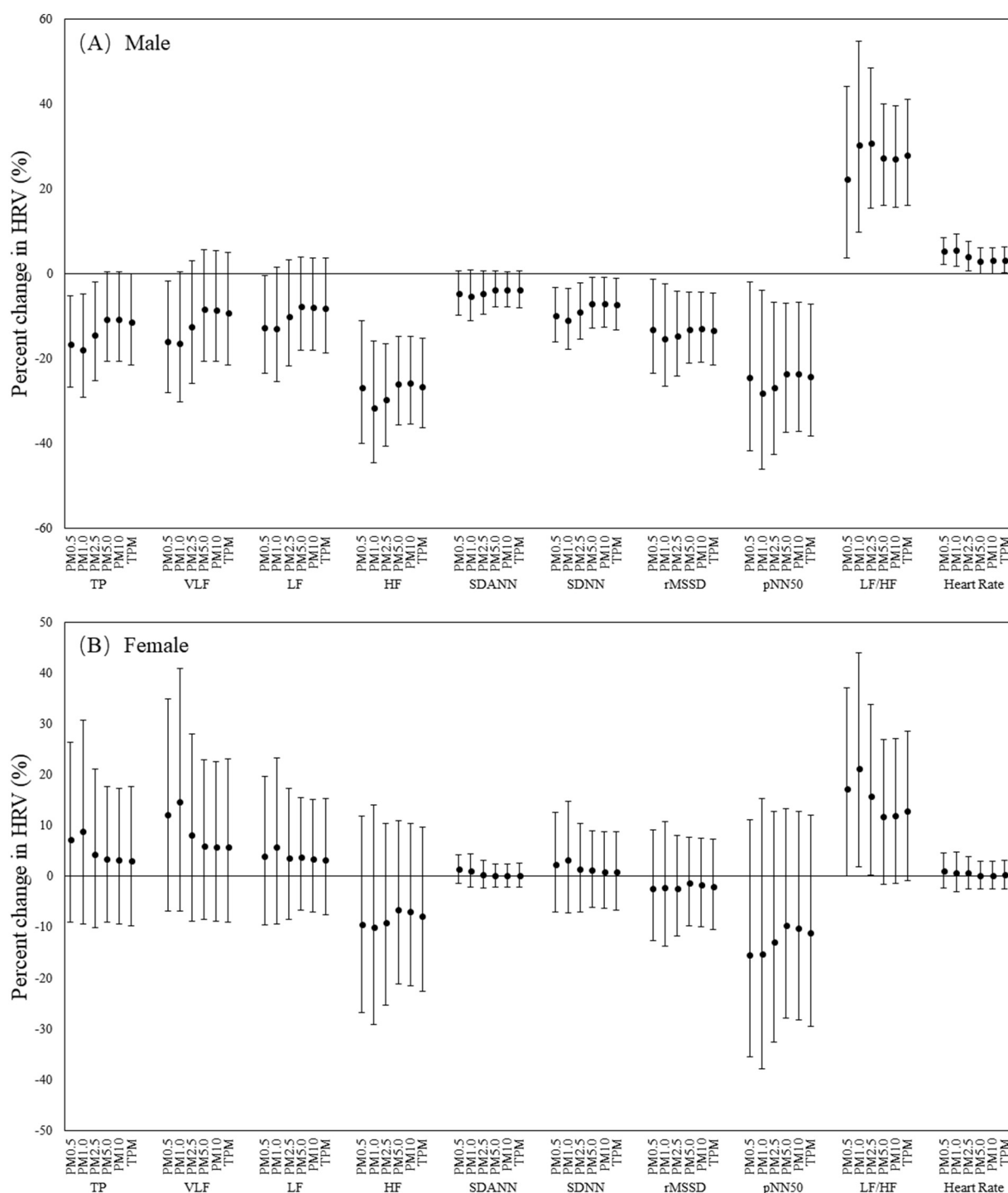


Fig. 1. Estimated percent changes with 95% confidence intervals in HRV indices and HR per interquartile range increase in size-fractioned PM. Estimates are adjusted for age, BMI, noise, temperature, relative humidity and the measured days.

et al., 2016). 8-OHdG is a sensitive biomarker of oxidative DNA damage caused by ROS. A report from the National Institute of Environmental Health Sciences (NIEHS) suggested that 8-OHdG in urine is the potential candidates for general biomarkers of oxidative stress (Kadiiska et al., 2005). Similar to our findings, Lin et al. found that urinary 8-OHdG concentrations was significantly associated with $PM_{2.5}$ levels; furthermore, there was a decrease trend in 8-OHdG (-37.4% ; 95% CI: -53.5 , -15.7) during the 2008 Beijing Olympic Games under the governmental air quality intervention (Lin et al., 2015). Our results found that another oxidative stress-related metabolite positively associated with PM in male is molybdopterin precursor Z, an important

active site for xanthine oxidoreductase (XOR) (Berry and Hare, 2004). The catalyse form of XOR plays an important role in the conversion of xanthine to uric acid, which contributes to the pathogenesis of hyperuricemia via XOR-derived ROS (Niu et al., 2017). Hyperuricemia is further strongly linked to hypertension, insulin resistance, obesity and hypertriglyceridemia, which each, and combined, eventually aggravate cardiovascular diseases (Battelli et al., 2018).

We found that citric acid cycle related metabolites were notably influenced by PM exposure with decreased level of α -ketoglutaric acid in males. α -ketoglutaric acid are important intermediates in citric acid cycle. The citric acid cycle is used to produce energy. It is the critical

Table 2

Metabolites identified as differentially expressed in participants between participants wearing with mask and without mask.

Metabolites	Molecule composition	*Trend	Fold change	p value	q value
Male					
Prolyl-arginine	C ₁₁ H ₂₁ N ₅ O ₃	↑	1.54	0.014	0.013
α-Ketoglutaric acid	C ₅ H ₆ O ₅	↑	1.50	0.040	0.083
Molybdopterine precursor Z	C ₁₀ H ₁₁ N ₅ O ₇ P	↓	0.61	0.046	0.067
8-Hydroxy-deoxyguanosine	C ₁₀ H ₁₃ N ₅ O ₅	↓	0.58	0.019	0.056
Female					
5-Hydroxylysine	C ₆ H ₁₄ N ₂ O ₃	↑	2.04	0.017	0.022
N-Acetylglutamine	C ₇ H ₁₂ N ₂ O ₄	↑	1.54	0.008	0.039
γ-Glutamyl ornithine	C ₁₀ H ₁₉ N ₃ O ₅	↓	0.65	0.022	0.026
2-Oxovaleric acid	C ₅ H ₈ O ₃	↓	0.56	0.018	0.022
5'-Phosphoribosyl-N-formylglycinamide	C ₈ H ₁₅ N ₂ O ₉ P	↓	0.52	0.049	0.072
Homovanillic acid	C ₉ H ₁₀ O ₄	↑	2.05	0.045	0.032
L-Citrulline	C ₆ H ₁₃ N ₃ O ₃	↑	1.79	0.010	0.032

Fold change was calculated by RIP/NIP; p value calculated by Student's *t*-test.

metabolic pathway of glucose, fat and amino acids, as well as the hub of the three major nutrients (Springsteen et al., 2018). Additionally, citric acid cycle, glycolysis and β-oxidation are known to be core pathways involved in energy production in the heart (West et al., 2016). Additionally, 2-oxovaleric acid, increased in NIP in females, is a kind of α-keto acid which can participate in both the citric acid cycle and glycolysis of human biological processes (Pudlik and Lolkema, 2013). Therefore, our data revealed the disorder of citric acid cycle and glycolysis could lead to an imbalance of energy supply after PM exposure.

Our previous animal study also found that exposure to PM_{2.5} resulted in elevating of citrate levels in the urine of rats, also suggesting that PM_{2.5}-exposure disturbs the citric acid cycle (Zhang et al., 2017). Citrate acid cycle is an important energy metabolism pathway which is regulated by glucose and insulin (Piloquet et al., 2003). Epidemiological studies had proposed that exposure to air pollutants may impair the insulin and glucose homeostasis, and increase the risk of obesity and metabolic syndrome (Wolf et al., 2016). Thus, the perturbed citric acid cycle could further lead to cardiac energy metabolism impairment, which is known to contributed to many cardiovascular pathologies, including atherosclerosis, ischemic heart disease and heart failure (Ussher et al., 2016). Thus, our study provides compelling evidences that confirms the interrelationship between metabolic syndromes and cardiovascular disease following exposure to air pollution levels.

Long-term oxidative stress or dysfunction of citric acid cycle can eventually trigger the cardiovascular events. As well as the immediate relationship with PM exposure, we also found several metabolites were definitely associated with cardiovascular effects associated with PM. 8-OHdG, a biomarker that is positively associated with severe cardiovascular diseases, such as hypertension, atherosclerosis, coronary artery disease and heart failure (Di Minno et al., 2016). Besides the obviously evaluated of 8-OHdG, the decreased levels of homovanillic acid and L-citrulline were detected in female urine of the NIP group (Table 2). Homovanillic acid, a kind of dopamine metabolite, can stimulate the vagus nerve to decrease the heart rate and blood pressure (Kolentini et al., 2013). L-citrulline is the end by-product of the production of nitric oxide (NO) from L-arginine. L-Arginine is known as to effectively increase the biological availability of NO and protect the vascular endothelial function (Curis et al., 2005). Clinical studies have reported that oral supplementation of L-citrulline could functionally reduce blood pressure and vascular conductance, balance autonomic nervous

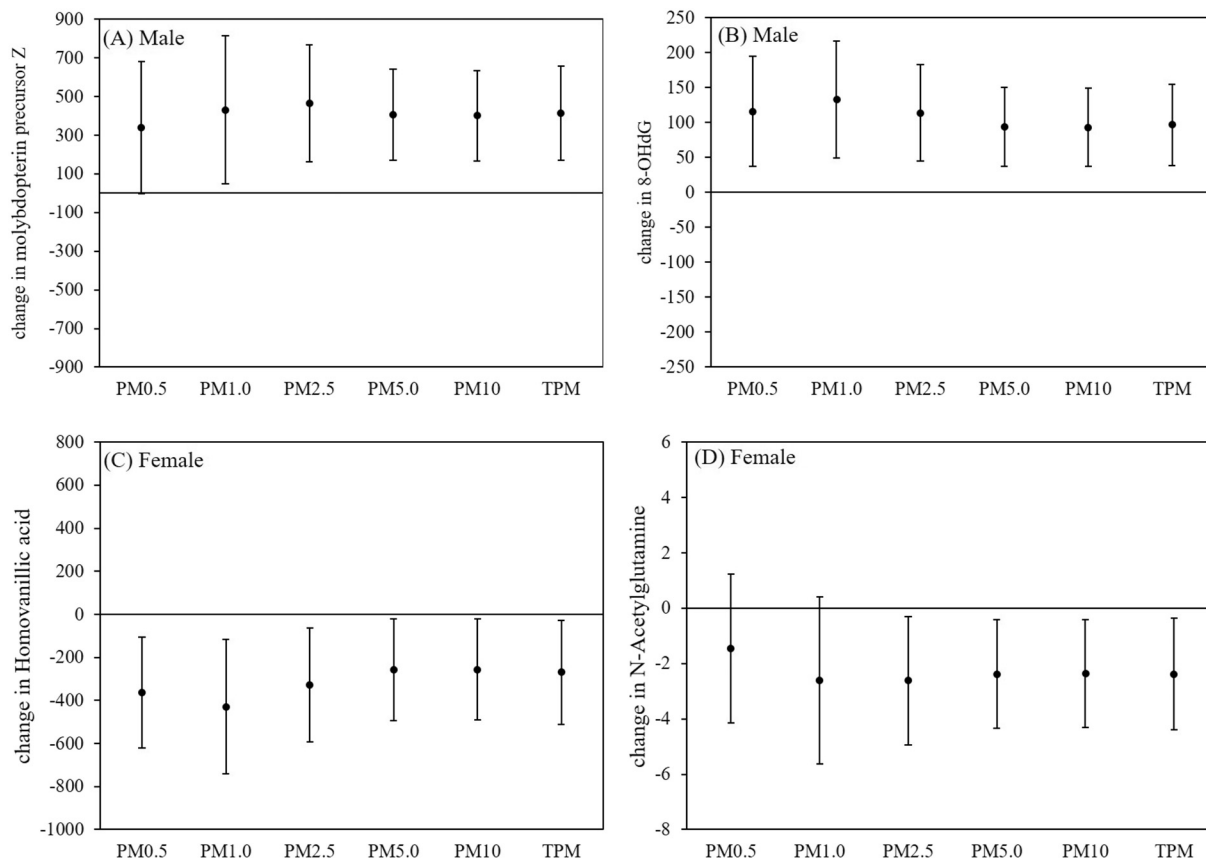


Fig. 2. Estimated percent changes with 95% confidence intervals in peak area of metabolites per interquartile range increase in size-fractioned PM. Estimates are adjusted for age, BMI, noise, temperature, relative humidity and the measured days.

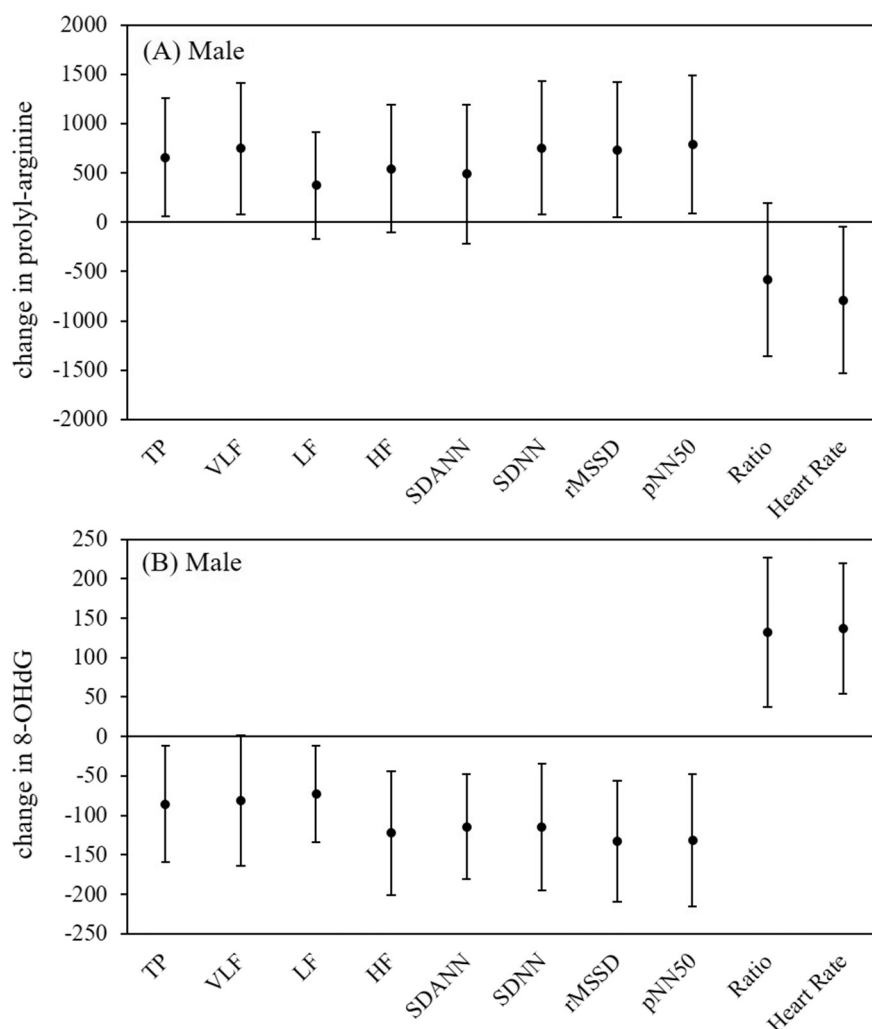


Fig. 3. Estimated percent changes with 95% confidence intervals in peak area of metabolites per interquartile range increase in HRV indices and HR. Estimates are adjusted for age, BMI, noise, temperature, relative humidity and the measured days.

system activity, as well as reducing the arterial stiffness and improving ventricular function (Alsop and Hauton, 2016). Therefore, our data indicates that short-term exposure to PM in a subway environment may increase the risk of cardiovascular disease, and wearing an efficient facemask could reduce the adverse effects caused by PM.

The linear mixed-effect models with a random intercept for each subject were performed to analyze the possible association between the size-fractionated PM and cardiovascular indicators (HRV and HR) or urine metabolites (Fig. 1 and Fig. 2). To our best knowledge, there are only few studies investigating multi-size of PM linked with cardiovascular effects (Dong et al., 2018), and no reports focusing on the association between urine metabolites and size-fractionated PM. Our data showed that the cardiovascular indicators of males were more sensitive to size-fractionated PM than those of females. Among them, LF/HF (representing sympathetic nerve function) has marked positive associations with all fractions of PM ($PM_{0.5}$, $PM_{1.0}$, $PM_{2.5}$, $PM_{5.0}$, PM_{10} and TPM); whereas HF (representing parasympathetic nerve function) is negatively associated with PM. This, indicates an imbalance occurred in autonomic nervous function with hypersensitivity of the sympathetic nerve and hyposensitivity of parasympathetic nerve in males. Although the blood pressure was slightly elevated in NIP compared with RIP, there were no statistically significant changes in either males or females (Table 1). In females, sympathetic nerve hypersensitivity also occurred only positively linked with $PM_{1.0}$. It was interesting to find that $PM_{1.0}$ was the greatest influential PM on indicators of cardiovascular effects in

both male and female groups. Similar results were found for associations between urine metabolites and different size-fraction of PM; the smaller the particle size (besides $PM_{0.5}$), the greater the effects on metabolites. The greatest increase in 8-OHdG and homovanillic acid were found to be induced by $PM_{1.0}$, indicating that $PM_{1.0}$ could be the most hazardous size-fraction for triggering cardiovascular effects. In the analysis of metabolites and indicators of cardiovascular effects, prolyl-arginine and 8-OHdG played opposite roles in regards to HRV and HR in males. Among all the metabolites, 8-OHdG was the most dominant one being positively associated with both size-fractionated PM and cardiovascular indicators.

In present study, we found that males could be more prone to cardiovascular effects than female after exposure to PM in subway environment, yet it was curious that metabolites were very different between male and female groups. The reason of this phenomenon could be due to the protection role of estrogen. Epidemiological studies have found that the incidence of cardiovascular disease among post-menopausal women is relatively higher than pre-menopausal women. Estrogen can activate endothelial nitric oxide synthase via estrogen receptors ER- α and ER- β to induce arterial vasodilation. Estrogen could also modulate serum lipid concentrations, coagulation and fibrinolytic systems, as well as antioxidant productions (Mendelsohn, 2002). Studies have found that a greater oxidative stress was occurred in healthy men compared to premenopausal women, which was attributed to estrogen (Ide et al., 2002). It has been indicated that estrogen scavenges

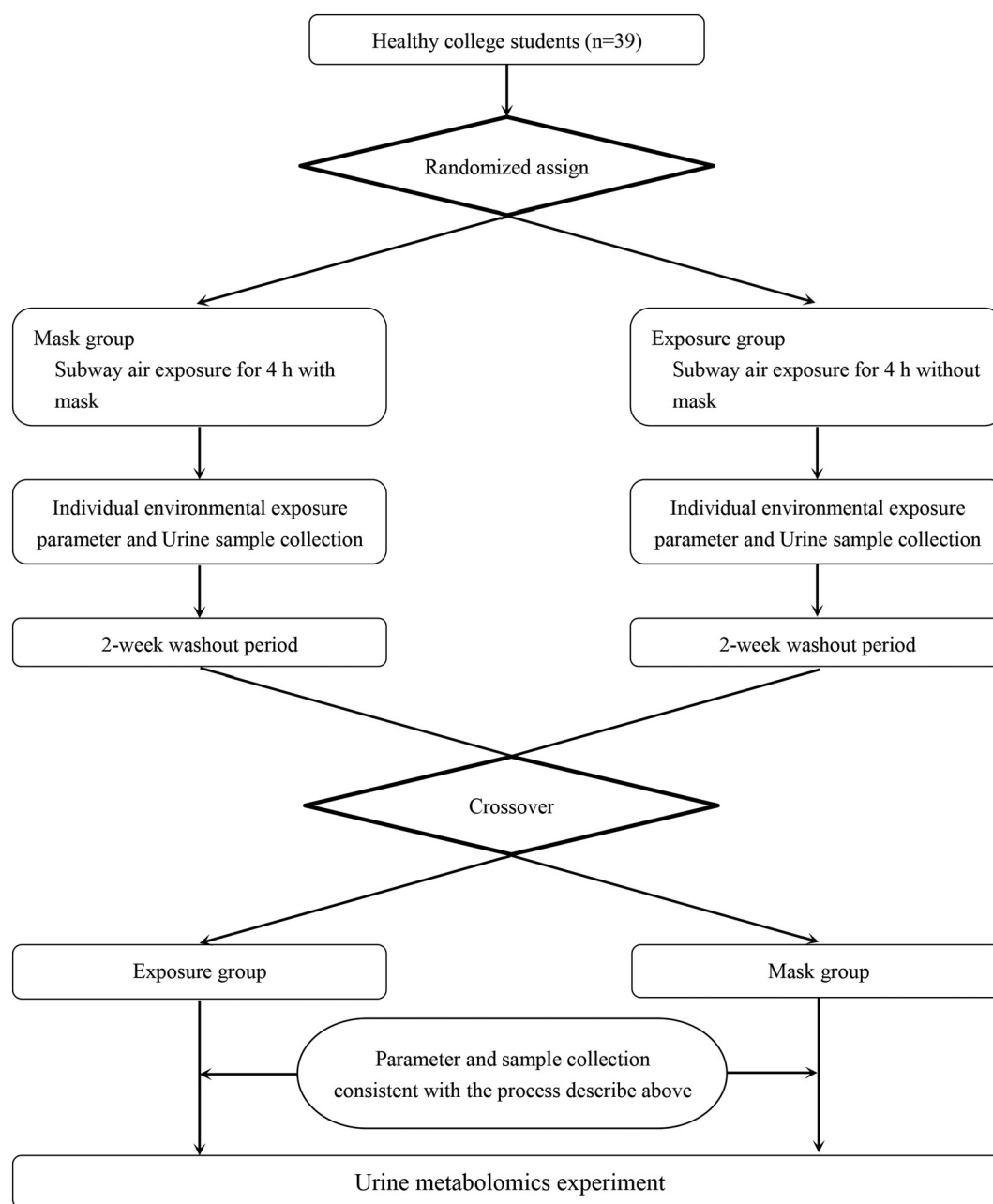


Fig. 4. Experimental design flowchart of a randomized crossover study.

free radicals in the presence of phenolic hydroxyl group, possibly through regulating NADPH-oxidase activity, e.g. the Nox1, Nox2 and Nox4 subunits. Indeed, results from animal experiment suggest that male rats have higher levels of Nox subunits, while female rats that undergone ovariectomy showed an increased NADPH-oxidase activity, which was attenuated by treatment with 17 β -estradiol (Kander et al., 2017). Thus, the great change in 8-OHdG between RIP and NIP only detected in males could be attributed to the antioxidant capacity of estrogen in females (Clegg et al., 2017).

The primary strength of this study is that we discovered 4 urine metabolites in males and 7 in females that could be used as potential biomarkers associated with PM-induced cardiovascular effects in subway system, and 8-OHdG was the most dominant one positive associated with both size-fractionated PM and cardiovascular effects. Secondly, this study observed a size dependent relationship between particle size and cardiovascular indicators as well as the urine metabolites, and PM_{1.0} is the greatest influential one among all the size-

fraction, which should be considered as the key prevention object. In addition, our results suggest that the cardiovascular effects of short-term exposure to PM in subway environment can be attenuated by wearing facemask. Yet, there were also some limitations in our study. The real PM exposure parameter in RIP was calculated according to filtration rate of respirator and not measured inside the respirator mask. In some previous studies, researchers have used particle counters to measure PM concentration levels in the ambient air and in the respirator mask to demonstrate that the mask could effectively reduce PM concentration (Langrish et al., 2009; Patel et al., 2016). Subjects were instructed to wear a respirator in a manner to ensure a close fit with their face and minimize the gaps between their face and the respirator. Thus we feel that the PM exposure parameter in RIP calculated according to filtration rate of respirator is credible. Finally, this study aimed to use non-target metabolomic analysis to preliminary explore the associations of metabolism and cardiovascular effects from exposure of size-fractionated particulate matter with and without respirator

intervention by detecting the urine metabolites, thus we didn't use standard references to confirm these tentatively identified metabolites, and it is required in the further confirmation studies.

5. Conclusion

In summary, our randomized crossover intervention study found that exposure to PM in subway system induced 4 differentially urine metabolites in males and 7 differentially urine metabolites in females. Among the 5 different sizes of PM investigated, PM_{1.0} was found to have the greatest influence on cardiovascular effects and metabolites both in male and female, and, in general, the smaller the particle size (besides PM_{0.5}), the greater effects on cardiovascular or metabolites changes. Among all the different metabolites, 8-OHdG was most prominently positively associated both with size-fractionated PM and cardiovascular indicators. In addition, males were more prone to trigger the cardiovascular events than females after exposure to PM in subway environment. The adverse cardiovascular effects could be effectively protected via a facemask intervention. Our study could provide a noninvasive way to identify specific urine metabolites that could predict individuals particularly susceptible to the adverse cardiovascular effects of subway PM.

Abbreviations

8-OHdG	8-hydroxy-deoxyguanosine
BP	blood pressure
ECG	Electrocardiogram
HR	heart rate
HRV	heart rate variability
HF	power in the high frequency band (0.15–0.4 Hz)
IQR	interquartile range
LF	power in the low frequency band (0.04–0.15 Hz)
MS ^E	mass spectrometry ^{Elevated Energy}
NIP	no intervention phase
PM	particulate matter
pNN50	percentage of number of NN interval with difference ≥ 50 ms
RH	relative humidity
RIP	respirator intervention phase
rMSSD	root mean square of successive differences between adjacent NN intervals
SDANN	standard deviation of the averages of NN intervals in all 5-min segments of the entire recording
SDNN	standard deviation of all NN intervals
Temp	real-time temperature
TP	total power
TPM	total particulate matter
UPLC/Q-TOF MS/MS	ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry
VLF	lower in the very low frequency band (0.01–0.04 Hz)

Declaration of Competing Interest

The authors declare they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2019.104920>.

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