



Cardiorespiratory Effects of Indoor Ozone Exposure Associated with Changes in Metabolic Profiles among Children: A Repeated-Measure Panel Study

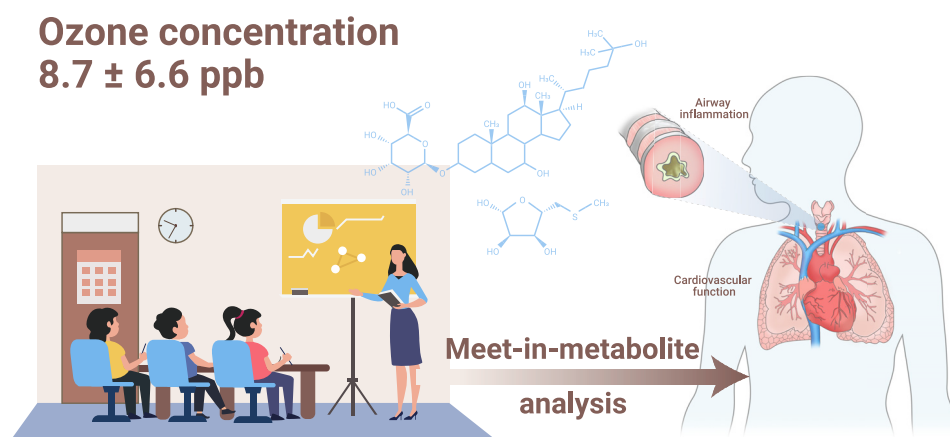
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Indoor O₃ exposure far below the indoor air quality limits disturbed amino acid and bile acid metabolism of children
- Exposure to indoor O₃ at low concentrations was associated with the deteriorated HRV, BP by affecting bile acid- and endogenous NO-related oxidation and inflammation
- Exposure to indoor O₃ at low concentrations was associated with the aggravated airway inflammation by reducing GPx-related anti-oxidation
- The cardiorespiratory effects of low-level ozone exposure indoors in children require additional attention
- Indoor ozone pollution should be controlled further and the current indoor ozone standards should be revised



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Ozone is one of the major gaseous pollutants associated with short-term adverse cardiopulmonary effects, even at concentrations below the current indoor air quality limits. However, the underlying biological mechanisms of cardiorespiratory changes with exposure to ozone remain unclear. To further explore molecular linkages between indoor ozone exposure and relevant cardiorespiratory effects, a repeated-measure panel study including 46 schoolchildren was conducted and real-time exposure measurements including ozone were performed inside classrooms every weekday during the study period. Repeated health measurements and urine sample collection were conducted in each participant. Ultra-high-performance liquid chromatography/tandem mass spectrometry and meet-in-metabolite approach were used in metabolomics analysis. Methods including mixed-effect models were adopted to identify metabolites associated with ozone exposure or health indices. Nine metabolites were found to be associated with ozone after mixed-effect model analysis, which are mainly involved in amino acid and bile acid metabolism. Boys may have a greater decrease in bile acid and RNA related metabolites. Four of the nine ozone-related metabolites were also associated with cardiorespiratory function indices. Furthermore, 26.67% of the positive association between ozone and heart rate was mediated by cholestane-3,7,12,25-tetrol-3-glucuronide. Exposure to ozone below the current indoor standards was associated with the deteriorated cardiovascular function by disturbing bile acid and endogenous nitric oxide-related oxidation and inflammation, and associated with the exacerbated airway inflammation by reducing GPx-related anti-oxidation. The results provide metabolic evidence of the cardiorespiratory effects of indoor ozone exposure. Indoor ozone pollution should be controlled further, and more attention should be paid to preventing its adverse health effects, especially in children.

KEYWORDS: indoor ozone; metabolomics; meet-in-metabolite approach; cardiorespiratory function; children

INTRODUCTION

Ozone is a highly hazardous gaseous environmental pollutant. Concentrations of ozone remain high in both higher-income and lower-/middle-income countries, and population-weighted ozone concentrations have increased.¹ Global deaths attributed to atmospheric ozone exposure amounted to 472,000 in 2019.² Both short- and long-term exposures to ambient ozone increase cardiorespiratory mortality and have been associated with deteriorated cardiopulmonary function in humans.^{3–5} Furthermore, most people have spent >80% of their time indoors, and indoor air pollution may have greater effects on health. However, there is little evidence showing the cardio-

respiratory effects of indoor ozone compared with those of particulate matter (PM), and the conclusions of a few related studies are inconsistent.

Some epidemiological studies have reported that indoor ozone exposure (200 ppb or 300 ppb) had no significant acute adverse cardiovascular consequences in young healthy adults.^{6,7} While our recent study suggested that short-term exposure to ozone (8.7 ± 6.6 ppb) indoors was associated with reduced cardiac autonomic and pulmonary function and increased fractional exhaled nitric oxide (FeNO) among children, its associations with pulmonary function and FeNO were not significant ($p > 0.05$).⁸ In accordance with our findings, one previous study also found that indoor ozone exposure of <10 ppb was not associated with lung function changes, while it was associated with platelet activation and blood pressure (BP) increase,⁴ which suggests that indoor exposure to ozone at relatively low concentrations (compared with the current standard limits and ranges of ozone concentrations in the studies conducted in the last decade; [Tables S1 and S2](#)) may possibly affect cardiovascular health. However, the exact mechanisms underlying the cardiorespiratory effects of ozone exposure remain uncertain.

At present, the possible mechanisms of the effects of ozone exposure on cardiorespiratory function include oxidative stress and airway inflammation. Several recent reviews have reported the pathophysiology to support the associations of adverse health effects with ozone and the chemical and toxicological properties of ozone as a strong oxidant, which could induce oxidative damage and immune-mediated inflammatory responses within and beyond the lungs.^{9,10} However, it is difficult to explore the systemic changes at the molecular level using traditional methods, and further studies are needed to confirm the mechanisms.

Metabolomics is a powerful tool in environmental health investigation that can identify the complex exposure biomarkers along with the changes in metabolic phenotypes related to the adverse outcomes in human populations. Therefore, metabolomics can improve the understanding of the underlying environmental causes of human health.¹¹ Some studies have adopted metabolomics to investigate the systemic metabolic alterations in response to air pollution, especially with regard to the cardiorespiratory effects of PM^{12–14}; however, there is far less evidence with regard to the cardiorespiratory effects of exposure to ozone. The limited ozone-related metabolomics studies proved that ambient ozone exposure was associated with changes in the concentrations of metabolites involved in creatine biosynthesis and fatty acid oxidation.^{15,16} A clinic-based crossover study reported increased levels of cortisol, corticosterone, and global lipid metabolism after 2-h exposure to ozone.¹⁷ Thus, metabolomics analysis can also be conducted to explore the molecular linkages between indoor ozone exposure and cardiopulmonary function as an intermediary tool. In addition, the abovementioned studies were primarily conducted in adults; children are potentially susceptible to environmental pollutants as their organs are not fully developed, and

Table 1. Baseline Characteristics of Participants

	Low Ozone Group (3.0–8.7 ppb)	Medium Ozone Group (9.1–11.4 ppb)	High Ozone Group (11.8–13.3 ppb)	p Value ^a
N ^b	44	46	33	
Sex, n (%)				0.907
Male	25 (56.8)	24 (52.2)	18 (54.5)	
Female	19 (43.2)	22 (47.8)	15 (45.5)	
Age, years				0.954
Mean ± SD	12.4 ± 0.6	12.4 ± 0.8	12.4 ± 0.7	
Range	11–14	11–14	11–14	
BMI, kg/m ²				0.053
Mean ± SD	17.8 ± 2.9	18.9 ± 3.3	19.7 ± 4.3	
Range	14.3–24.0	14.7–32.8	14.5–32.8	

Abbreviations: BMI, body mass index; SD, standard deviation.

^aThe comparisons of baseline characteristics of participants among three groups were conducted with one-way ANOVA (numerical variables) and chi-square test (categorical variables).

^bObservations after excluding all missing values and abnormalities.

may even be susceptible to ozone at low concentrations. Impaired cardiorespiratory function during childhood can increase the related disease risk later in life.¹⁸

Therefore, metabolomics analysis was performed on urine samples collected from a repeated-measure panel study to explore the metabolic linkages between exposure to natural indoor ozone and cardiorespiratory effects in children via meet-in-metabolite approach (MIMA).

RESULTS

Participants' Characteristics

Forty-six children including 24 (52%) boys and 22 (48%) girls completed the study. The participants' age was 12.4 ± 0.8 years, and the body mass index (BMI) was 18.8 ± 3.4 kg/m². The baseline characteristics of participants in three groups are presented in Table 1.

Description of Indoor Exposure Measurements

Overall, the average concentrations of indoor ozone, fine particles (PM_{2.5}), inhalable particles (PM₁₀), and black carbon (BC) were 8.7 ± 6.6 ppb, 75.4 ± 53.8 µg/m³, 563.9 ± 498.3 µg/m³, and 4.4 ± 3.3 µg/m³, respectively. All participants spent >80% of their time in the classrooms during the study period according to their self-reported activity diaries.

Description of Cardiorespiratory Function Measurements

The averages of some representative cardiorespiratory indices were as follows: SD of all normal-to-normal intervals (SDNN), 65.7 ± 23.3 ms; power in high frequency (HF), 381.6 ± 355.6 ms²; the ratio of power in low frequency (LF) to power in HF (LF/HF), 3.9 ± 3.2; heart rate (HR), 90.3 ± 12.9 min⁻¹; systolic BP (SBP), 105.4 ± 7.3 mm Hg; diastolic BP (DBP), 64.2 ± 6.0 mm Hg; FeNO, 17.2 ± 7.2 ppb; forced expiratory volume in 1 s (FEV₁), 2.2 ± 0.5 L; and peak expiratory flow (PEF), 342.6 ± 79.4 L/min. Furthermore, the trend test analysis (Figure S1) implied that, with the increase in indoor ozone exposure, the heart rate variability (HRV) indices (HF, LF, SDNN, and the square root of the mean squared differences between adjacent normal-to-normal intervals [rMSSD]) showed downward trends, while LF/HF, HR, ST-segment elevations, and BP increased, suggesting the deterioration in the cardiovascular function after exposure to ozone. In addition, FeNO increased, while lung function showed a downward trend. The results were in accordance with those of a previous publication,⁸ although only HF, SDNN, LF/HF, HR,

FeNO, and V2_ST segment elevation associated with ozone exposure showed significant trends in the current analysis.

Identified Urinary Metabolites

Scoring plots generated from the partial least-squares discriminant analysis (PLS-DA) model presented good separations of the metabolic profiles, which were characterized by different levels of ozone exposure (Figure 1). The PLS-DA model was validated using a strict permutation test (999 random permutations), and no overfitting was observed (Figure S2), indicating that the model for discriminating ozone exposure differences was robust. There were 25,675 compounds (negative ion mode, 11,385; and positive ion mode, 14,290) detected in the non-targeted metabolome analysis via ultra-high-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS), which measured the relative abundance. Then 51 compounds with variable importance in projection (VIP) of >2 and significant abundance changes (p < 0.05) between high and low ozone concentration groups were screened to determine the separation of urinary metabolite profiles. Finally, nine ozone-related metabolites were identified through the mixed-effect model analysis and identified by searching the Human Metabolome Database (HMDB) (Table 2). Six of the nine metabolites were negatively associated with ozone exposure concentrations, while the other three were positively associated with ozone concentrations.

Analysis of the exposure-response relationships also showed metabolite changes according to sex. No significant difference was observed in most of the metabolite-ozone relationships between boys and girls, except for cholestane-3,7,12,25-tetrol-3-glucuronide (p < 0.01) and N4-acetylcytidine (p < 0.04) (Figure 2). Boys showed a greater decrease in the levels of these two metabolites with an interquartile range (IQR) increase in ozone exposure. The interaction analysis of BMI (cut off by the median: 18.29 kg/m²) showed that BMI and ozone had no significant interaction on the metabolites (Table S3).

Figure 3 further presents the different lag time effects of ozone exposure on metabolites. Most metabolites, such as 5-methylthioribose, cholestane-3,7,12,25-tetrol-3-glucuronide, 4-methylumbelliferone, argininosuccinic acid, 3,4-dihydroxymandelic acid, and oxoglutaric acid, were immediately affected by ozone exposure. However, the levels of some metabolites showed significant changes after exposure to ozone over longer lag times, especially 3'-O-methyl(-)-epicatechin. Most metabolites showed no significant changes at lag 01 day, while some results reversed at lag 02 day compared with those at lag 0 day.

In addition, four cardiorespiratory-function-related compounds were identified using the linear mixed-effect model. In Table 3, cholestane-3,7,12,25-tetrol-3-glucuronide was negatively associated with HR, oxoglutaric acid was positively associated with LF/HF, and oxoglutaric acid and 5-methylthioribose were both positively associated with DBP. 4-Methylumbelliferone was negatively associated with FeNO. However, none of the metabolites were associated with lung function. The analysis of the lag effects of metabolites on cardiorespiratory function indicated that the associations between metabolites and BP may reverse at lag 2 day, and some significant associations between metabolites and HRV or ST-segment elevations had hysteresis effects (Figures S3–S5).

Metabolic Relation of Ozone Exposure to Cardiorespiratory Function

Table 2 presents the indoor ozone-related metabolites. Nine ozone-related metabolites belong to four major metabolisms and seven specific pathways, including amino acid (methionine, arginine, glutathione, and tyrosine) metabolism, bile acid secretion, epicatechin degradation, and ribonucleic acid (RNA) degradation. Regarding the cardiovascular indices, HRV-related metabolites belong to arginine metabolism and bile acid secretion; BP-related ones belong to arginine and methionine metabolism. In addition, the respiratory inflammation-related metabolite belongs to glutathione metabolism (Table 3).

A biological pathway analysis of the metabolites was further conducted and eight metabolites were integrated into a metabolic network (Figure S6), which involved amino acid metabolism, bile acid secretion, RNA degradation,

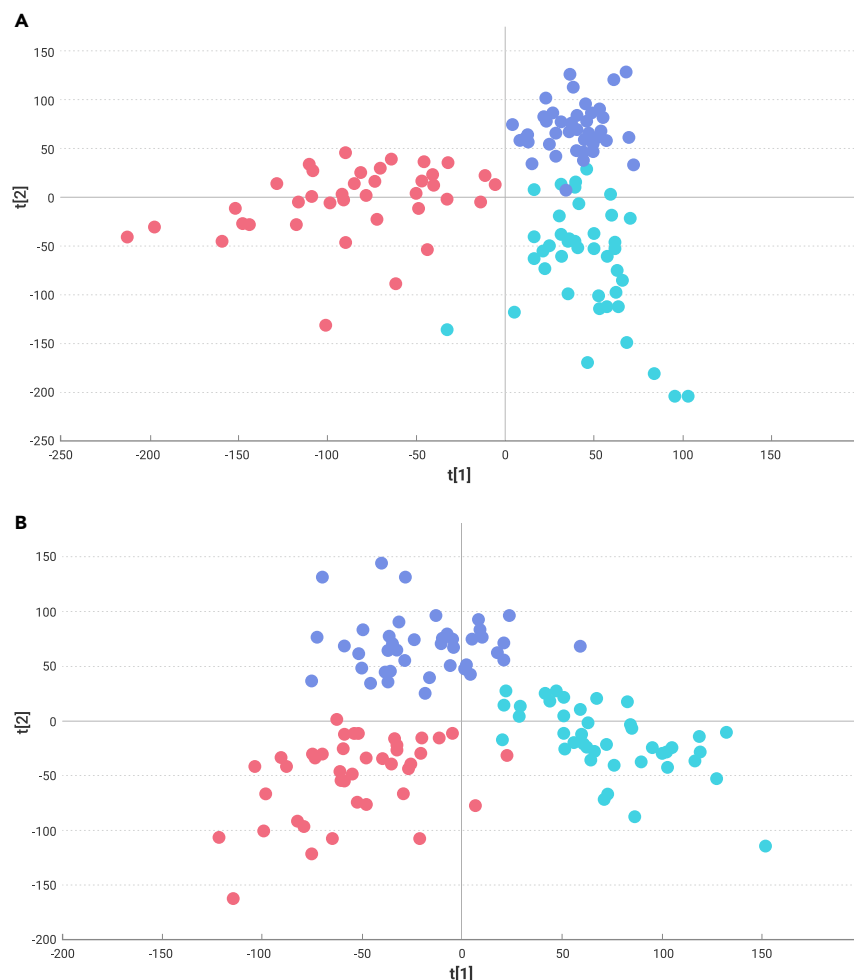


Figure 1. Scoring Plots of the PLS-DA Model with Ozone Concentrations by Tertiles as the Classifier (A) Negative ion mode, ● low ozone concentration group, ● medium ozone concentration group, ● high ozone concentration group; (B) positive ion mode, ● low ozone concentration group, ● medium ozone concentration group, ● high ozone concentration group. PLS-DA, partial least-squares discriminant analysis.

and epicatechin degradation. These metabolic pathways may indicate the deterioration of energy production, inflammation, and oxidation reactions after exposure to ozone.

Receiver Operating Characteristic Analysis

The receiver operating characteristic (ROC) curve is widely used to evaluate the diagnostic performance of metabolites. The closer the area under the curve (AUC) value approaches to 1, the better diagnostic performance the metabolite provides. Table 2 shows that four of the nine ozone-related metabolites had AUC values >0.7 , respectively, which indicates that the metabolites have moderate to high discriminating abilities for ozone exposure. A multiple metabolite model may provide a better discriminating capability compared with a single one.¹² The combinations of eight (excluding oxoglutaric acid) or both nine ozone-related metabolites (with AUC = 0.893) were the best indicators for ozone exposure compared with other combined metabolite patterns (Figure 4). A confusion matrix can show the predictive accuracy as the percentage of correctly classified samples in a given class. For the model, the predictive accuracies were calculated as 91.4% and 80.9% for the lowest (\leq first tertile) and highest ($>$ third tertile) ozone concentration group, respectively (Figure 4).

Metabolites Mediating the Association of Ozone Exposure with Cardiorespiratory Function

The mediation effects of the metabolites on the associations of ozone exposure with cardiorespiratory function were further calculated by conducting a causal mediation analysis. Among the metabolites, 26.67% of the positive association between ozone and HR was mediated by cholestan-

3,7,12,25-tetrol-3-glucuronide (indirect effect = 0.12, $p = 0.04$). As the previous study showed that ozone exposure at lag 0 day had no associations with LF/HF, BP, FeNO, pulmonary function, V2_ST, and V5_ST-segment elevations,⁸ a mediation analysis was not conducted. In addition, 23 metabolites had non-significant mediation effects ($q > 0.05$) on the associations of ozone with other HRV and ST segment elevation indices (Table S4).

DISCUSSION

Although the statistical associations have been widely investigated, the molecular linkages between indoor ozone exposure and cardiorespiratory function still remain unclear. The present study identified a set of metabolites associated with indoor ozone exposure by conducting an untargeted metabolomics analysis. They are mainly involved in amino acid and bile acid metabolism. Ozone exposure was associated with the disturbed cardiovascular function and airway inflammation by aggravating metabolism-related oxidation and inflammation in children. Although the associations of ozone exposure with the subclinical indices of BP and airway inflammation responses were not considered significant,⁸ perturbations were observed in BP- and FeNO-related metabolic pathways at the molecular level in the current study. The indoor ozone concentration below the existing 1-h and 8-h standard limits (Table S1) was still found to be associated with adverse effects on cardiopulmonary function and metabolic disturbance among children. It is necessary to revise the current indoor ozone standards by setting the 8-h standard limit for the classroom environment and raising the limit level. Our previous studies also explored the associations of ozone exposure with cardiopulmonary function among patients with chronic obstructive pulmonary disease, and healthy older and younger adults,^{19–21} whereas per IQR

Table 2. Major Associations of Indoor Ozone Exposure with Metabolites

Metabolism	Pathway	Compound Name	VIP	Fold Change		AUC ^a	Percentage Change (95% CI) in Metabolites ^b
				M/L	H/L		
Amino acid metabolism	methionine pathway	5-methylthioribose	3.17	1.76	11.06*	0.634 (0.511–0.757)*	3.23(0.93, 5.57) [#]
		S-adenosylmethionine	2.02	0.24*	0.13**	0.761 (0.653–0.869)**	–2.60(–4.45, –0.72) [#]
	arginine pathway	argininosuccinic acid	2.20	0.18**	0.18**	0.720 (0.607–0.834)**	–4.34(–6.49, –2.14) [#]
		oxoglutaric acid	2.29	1.44**	5.95*	0.644 (0.524–0.763)*	2.99(0.93, 5.10) [#]
	glutathione pathway	4-methylumbelliferone	2.11	0.25*	0.43*	0.638 (0.519–0.758)*	–3.29(–5.92, –0.59) [#]
	tyrosine pathway	3,4-dihydroxymandelic acid	2.06	0.31**	0.19**	0.702 (0.585–0.819)**	–2.71(–5.10, –0.25) [#]
Bile acid secretion	bile acid secretion	cholestane-3,7,12,25-tetrol-3-glucuronide	2.04	0.35	0.36**	0.671 (0.554–0.788)**	–4.13(–6.77, –1.43) [#]
RNA metabolism	RNA degradation	N4-acetylcytidine	2.20	0.17**	0.15**	0.753 (0.649–0.856)**	–3.02(–5.01, –0.98) [#]
Epicatechin degradation	epicatechin pathway	3'-O-methyl(–)-epicatechin	2.04	1.17	5.67**	0.698 (0.584–0.811)**	5.18(2.86, 7.56) [#]

Abbreviations: AUC, area under the curve; CI, confidence interval; H, high ozone group; L, low ozone group; M, medium ozone group. RNA, ribonucleic acid; VIP, variable importance in projection.

* $p < 0.05$; ** $p < 0.01$; [#] $q < 0.05$.

^aAUC derived from ROC analysis.

^bPercentage change refers to the change ranges of metabolites with per IQR increase of ozone (10.9 ppb). Adjusted for age, sex, BMI, class and long-term time trend (including day of measurement), BC, temperature, RH, and noise, and includes the number of participants as a random effect.

increase of ozone exposure at 1-day moving average was associated with less changes in cardiorespiratory function among these populations compared with children. Thus, the cardiorespiratory effects of ozone exposure in children require additional attention.

Biological Significance of the Indoor Ozone Exposure-Related Metabolites

Some epidemiological studies have reported the associations between ozone exposure and human metabolism, whereas the reported ozone exposure levels were relatively high and have shown inconsistent findings. One study reported that increased concentrations of cortisol, corticosterone, and global lipid metabolism were associated with ozone exposure,¹⁷ while two studies proved that ozone exposure was associated with changes in the concentrations of metabolites involved in creatine biosynthesis and fatty acid oxidation.^{15,16} The current study found that nine metabolites were associated with the indoor ozone exposure below the current indoor air quality standards, mainly involving seven metabolic pathways.

Oxoglutaric acid increased with ozone exposure, which implied a reduction in arginine metabolism, with the argininosuccinic acid levels decreased. Arginine is the only nitrogen supply precursor of endogenous nitric oxide (NO) and the key information molecule involved in the physiological and pathological processes. The disturbance in arginine generation caused by ozone exposure reported in this study is in line with the recent observations that indicated that exposure to ozone could increase the risk of cardiopulmonary disease with decreased arginine by promoting inflammation and oxidative stress.^{15,22} The downregulation of 3,4-dihydroxymandelic acid indicated the association of tyrosine accumulation with ozone exposure. Tyrosine increased after repeated ozone exposures.²³ Several *in vivo* studies also reported that ozone exposure can cause the imbalance of oxidative and nitrate stress as the tyrosine increase.^{24,25} Reduction in S-adenosylmethionine and increase in 5-methylthioribose suggest the downregulation of methionine, which is important for anti-oxidation. The current finding implied that indoor ozone exposure may be associated with the downregulation of methionine and aggravation of oxidative stress. 4-Methylumbelliferone,

involved in the glutathione metabolism, was negatively associated with ozone exposure. Its decrease indicated a reduction in the anti-oxidation capacity. The result of a previous study is in accordance with the present result, in which reduction in glutathione peroxidase (GPx) activity indicated the decrease in antioxidants.²⁶

Cholestane-3,7,12,25-tetrol-3-glucuronide is an HMDB predicted metabolite.²⁷ A decrease in cholestane-3,7,12,25-tetrol-3-glucuronide may imply an imbalance in bile acid metabolism. Bile acid is an important component in bile and plays an essential role in lipid metabolism, and its downregulation indicates the impaired lipolysis. This finding is consistent with that of a study conducted in rats, which demonstrated the aggravation of oxidative stress with bile acid reduction.²⁸ On the other hand, the metabolite may be the response of ozone reacting with cholesterol, which can yield a complex series of products. Ozone specifically cleaves the $\Delta 5,6$ double bond of cholesterol to yield 5-hydroperoxy-B-homo-6-oxa-cholestan-3 β ,7 α -diol, which can further react to the primarily reductant of 5,6-secosterol or aldol condensed to norcholestan-6-carboxal in solution. It can also be epoxidized into 5 β ,6 β -epoxycholesterol and further produce cholestan-6-oxo-3 β ,5 α -diol with minor cholestan-3 β ,5 α ,6 β -triol in the lipid environment.^{29–31} Interestingly, cholestan-6-oxo-3 β ,5 α -diol is the only metabolite that can significantly mediate the ozone effect on HR, which would guide further studies on the urinary biomarkers of ozone exposure and cardiovascular effects.

In addition to amino acid and bile acid metabolism, ozone exposure was also associated with epicatechin degradation and RNA degradation. The increase in 3'-O-methyl(–)-epicatechin suggested the enhancement in epicatechin degradation. Epicatechin could inhibit inflammation and oxidative and endoplasmic reticulum stress, modulate mitochondrial biogenesis and function, and regulate events in the gastrointestinal tract and the pancreas that affect glucose homeostasis.^{32,33} Although no evidence directly supports this result, a recent study demonstrated that catechin could enhance ozone removal through a balanced redox state in plant cells.³⁴ The ozone-related epicatechin reduction implied the aggravation of oxidative stress, inflammation, and energy disorder. In addition, the decrease in N4-acetylcytidine indicated a decrease in mRNA acetylation,

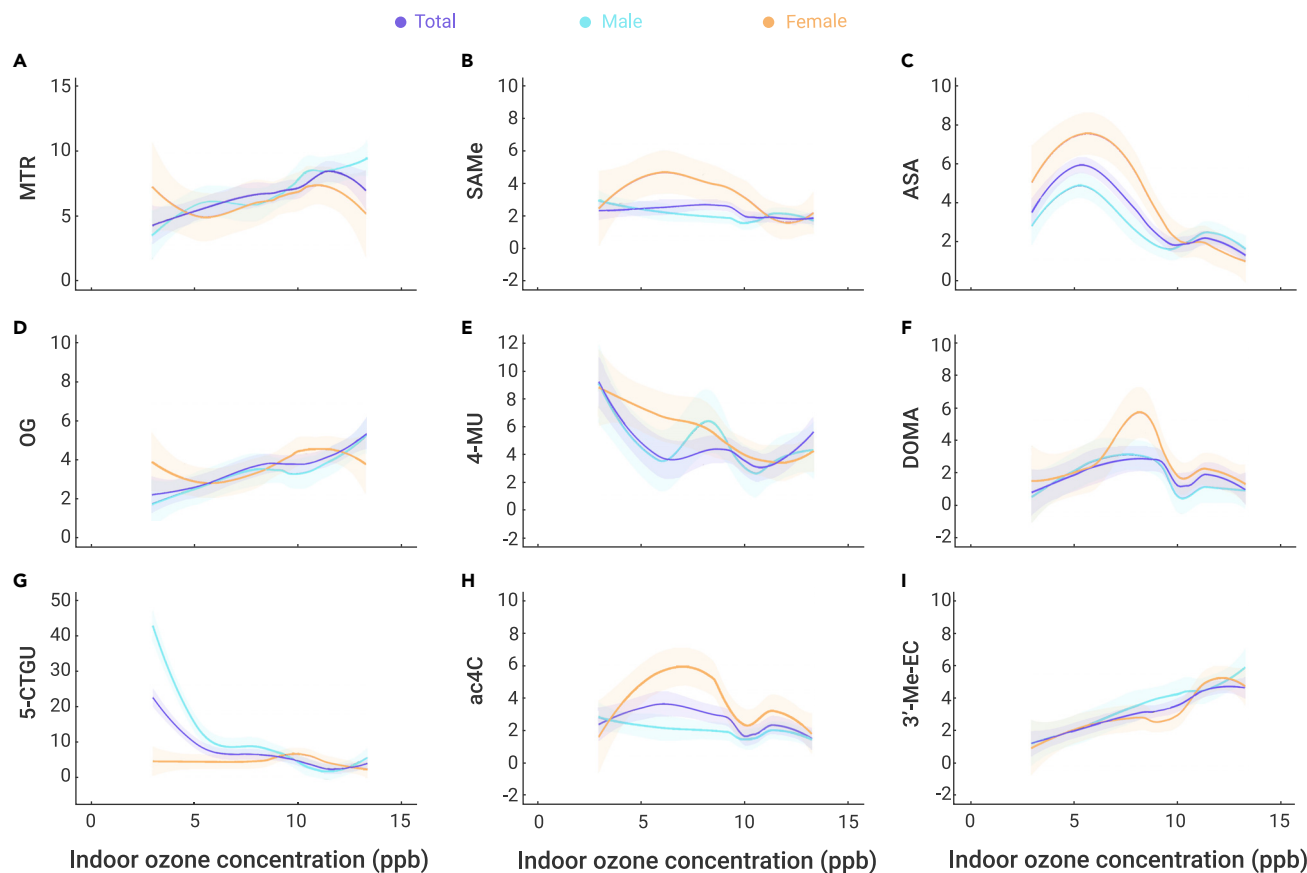


Figure 2. Exposure-Response Relationships between Indoor Ozone Exposure and Metabolites Linear mixed-effect models were conducted for exposure-response relationship analysis, where y was the logarithm of the relative peak intensity of the identified metabolites and x referred to indoor ozone concentrations with the same covariates mentioned in the statistical analysis. Then y was exponentially converted to return to the original measurements of the relative abundance of metabolites when the figure was drawn. The unit of y axis is relative abundance. MTR, 5-methylthioribose; SAME, S-adenosylmethionine; ASA, argininosuccinic acid; OG, oxoglutaric acid; 4-MU, 4-methylumbelliferone; DOMA, 3,4-dihydroxymandelic acid; 5-CTGU, cholestane-3,7,12,25-tetrol-3-glucuronide; ac4C, N4-acetylcytidine; 3'-Me-EC, 3'-O-methyl(-)-epicatechin.

reflecting the insufficient adenine nucleoside triphosphate (ATP) energy supply and reduction in translation efficiency.^{35,36}

In summary, indoor ozone exposure even below the current indoor air quality standards was associated with the disorder in amino acid metabolism, bile acid secretion, epicatechin degradation, and RNA degradation, which were related to oxidative/nitrative stress, inflammation, and energy imbalance. Their disturbance may further adversely affect cardiopulmonary development in children. These results are supported by those of previous studies.^{9,10,15,37,38} One recent study indi-

cated that the concentrations of proinflammatory cytokines were significantly and negatively associated with 12-h ozone exposures and positively associated with 2-week ozone exposures in healthy adults.³⁹ This may be attributed to the greater sensitivity of children, whose inflammatory responses may appear earlier.

Two other observations are also worth noting. Almost all metabolites can change significantly within a short period of time after ozone exposure (lag 0, 2, 4, 6, 8 h). The greatest effect time of the associations between ozone exposure and metabolites varied, which suggested that different metabolic

Table 3. Major Associations of Ozone-Related Metabolites with Cardiorespiratory Function

Metabolism	Pathway	Compound Name	Health Indicators	Percentage Change (95% CI) in Health Indicators ^a
Amino acid metabolism	arginine pathway	oxoglutaric acid	LF/HF	0.050 (0.002, 0.100) [#]
			DBP	0.040 (0.010, 0.070) [#]
	methionine pathway	5-methylthioribose	DBP	0.010 (0.001, 0.020) [#]
	glutathione pathway	4-methylumbelliferone	FeNO	-0.340 (-0.670, -0.010) [#]
Bile acid secretion	bile acid secretion	cholestane-3,7,12,25-tetrol-3-glucuronide	HR	-0.020 (-0.050, -0.003) [#]

Abbreviations: CI, confidence interval; DBP, diastolic blood pressure; FeNO, fractional exhaled nitric oxide; HR, heart rate; LF/HF, the ratio of power in low frequency to power in high frequency.

[#] $q < 0.05$.

^aPercentage change refers to the change ranges of health indices with per IQR increase of relative abundance of the metabolites. Adjusted for age, sex, BMI, class and long-term time trend (including day of measurement), BC, temperature, RH, and noise, and included the number of participants as a random effect. IQR: 5-methylthioribose, 5.76; cholestane-3,7,12,25-tetrol-3-glucuronide, 4.08; 4-methylumbelliferone, 8.79; oxoglutaric acid, 3.21.

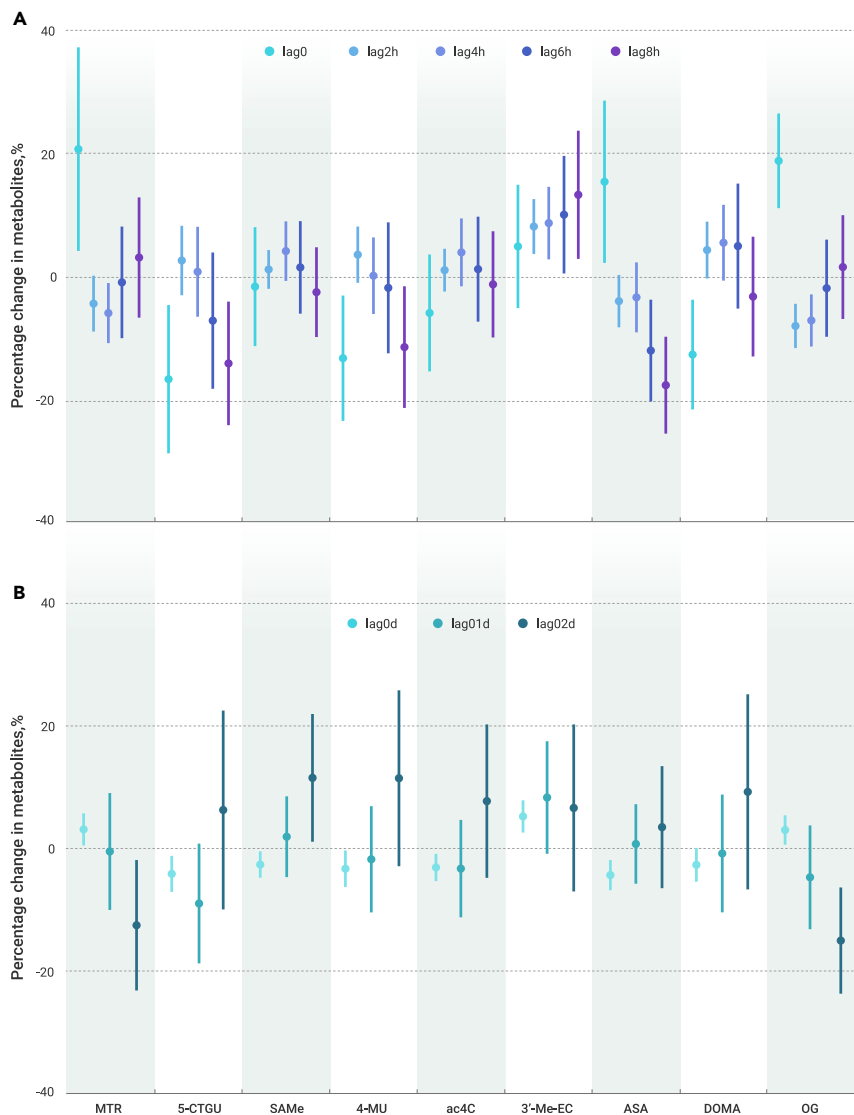


Figure 3. Estimated Changes in the Metabolites with IQR Increases of Ozone at Different Metrics in Participants (A) Changes over moving average of different lag times on the day of urine collection; (B) Changes over moving average of different lag days. Estimated changes were presented with mean and 95% confidence intervals. The IQR of ozone was 10.9 ppb. Abbreviations as in Figure 2.

pathways have different susceptibilities. The appropriate time should be considered when collecting urine samples according to the study purpose and requirements. On the other hand, boys may exhibit greater changes in cholestane-3,7,12,25-tetrol-3-glucuronide and N4-acetylcytidine with exposure to ozone. Moreover, BMI and ozone exposure did not show any interaction on the metabolites. There are two possible speculations: firstly, the metabolic changes caused by ozone exposure may indeed not differ between different BMIs, or the interaction may exist, whereas it has not been presented due to the very limited range of BMI in the present study. However, neither sex nor BMI was found to have interactions with ozone exposure indoors on cardiorespiratory function among children (data not shown). In general, all the speculations mentioned above need further validation regardless of sex or BMI, as the sample size is relatively small.

Predictabilities of Metabolites to the Ozone Exposure

ROC curve analysis is an objective and statistically valid method for defining the clinical utility of a biomarker. A biomarker with an AUC of >0.7 is usually acceptable for most clinical applications.^{40,41} In the current study, the combinations of eight or nine metabolites (AUC = 0.893) showed great capabilities in the prediction of ozone exposure. Taken together, a series of metabolites identified in this study were proposed to have greater potentials for evaluating indoor ozone exposure even below the current indoor air quality standards, and the metabolite predictabilities were sensitive and specific.

Metabolites Mediating the Effects of Ozone Exposure on Cardiorespiratory Function

The identified cardiorespiratory-function-related metabolites were mainly associated with bile acid secretion and arginine, methionine, and glutathione metabolism. Disorders in bile acid metabolism cause cardiovascular diseases,⁴² which is in accordance with the finding that the reduction of bile acid and disturbance of cholesterol reaction (reflected by the decreased cholestane-3,7,12,25-tetrol-3-glucuronide) were associated with the adverse effect on HR. The causal mediation analysis further indicated that 26.67% of the positive association between ozone and HR was mediated by cholestane-3,7,12,25-tetrol-3-glucuronide, which strengthened the causal relationship. This metabolite also had non-significant mediation effects on the associations of ozone with power in total frequency (TP), HF, LF, SDNN, and IL_ST segment elevation, suggesting that ozone exposure may affect HRV and ST-segment elevations with disturbing the bile acid and cholesterol metabolism. Besides, S-adenosylmethionine and 4-methylumbelliferone were the two major metabolites that had no significant mediation effects on the negative associations between ozone and most HRV indices (Table S4). These results suggest that ozone exposure may lower HRV by reducing the anti-oxidation reaction, which is consistent with the findings of a previous study demonstrating that associations of air pollution with HRV were mediated by oxidative stress pathways.⁴³ However, the non-significant results need further verification.

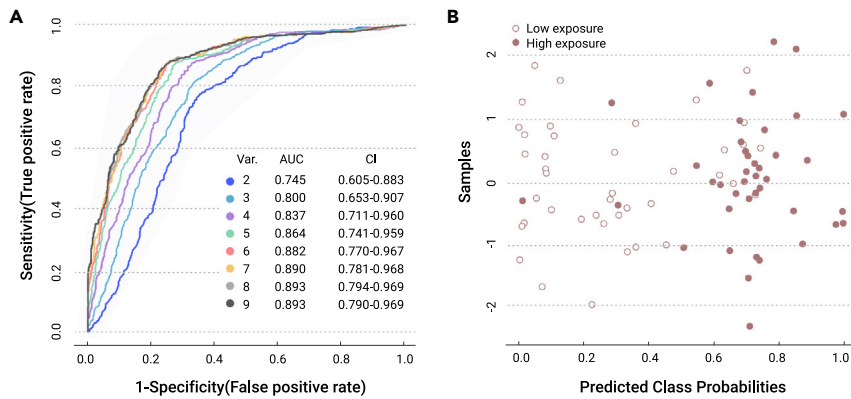


Figure 4. ROC Curves (A) Probability views and (B) confusion matrix of the combined metabolite patterns for ozone. ROC curves are generated by Monte Carlo cross-validation using balanced subsampling. The predicted class probabilities were calculated for each sample using the developed ROC models. AUC, area under the curve; CI, confidence interval; Var., variable (i.e., metabolite) numbers.

On the other hand, the reduction in arginine (indicated by the increase in oxoglutaric acid) may suggest the over-activation of arginase, which is related to the dysfunction and pathologies of the cardiovascular system, including HRV and DBP.⁴⁴ DBP was also positively associated with 5-methylthioribose, whose increase indicates downregulation of methionine. A recent study also reported a similar result, indicating that methionine was downregulated in essential hypertension patients.⁴⁵ In addition, one index of airway inflammation, FeNO, was negatively associated with 4-methylumbelliferone. The negative association is related to glutathione metabolism, and its decrease indicates the reduction of glutathione, an important anti-oxidant. The metabolic process suggests the deterioration of oxidative stress, which may be related to airway inflammation. Results showed that these metabolic pathways may have partial mediating effects in the association between indoor ozone exposure and cardiovascular function, airway inflammation, which are associated with oxidative stress and inflammation (Figure 5). Oxidative stress and systematic inflammation are the two common pathophysiologic mechanisms of cardiopulmonary diseases,^{46,47} which may support the results.

Strengths and Limitations

To the best of our knowledge, this is the first epidemiological study to investigate the potential metabolic linkages between indoor ozone exposure and cardiorespiratory function. Ozone exposure and cardiorespiratory-function-related metabolites are associated with oxidative/nitrative stress, inflammation, and energy imbalance, which have linked the exposure-outcome pathway via the MIMA strategy. Secondly, repeated urine sample collections and pollution measurements could decrease the exposure misclassification and estimation biases for certain associations. In addition, children are susceptible and their metabolism may be different from that of adults as their organ systems are immature. The study could bring new mechanistic insight in protecting children's health from indoor air pollution.

The current study has some limitations. Firstly, although some significant associations have been observed, the sample size is relatively small, which could weaken the statistical correlations. To mitigate the effect of the sample size on statistical power, the associations between ozone exposure and cardiorespiratory function, ozone exposure and metabolites, cardiorespiratory function and metabolites, and the mediation effects of metabolites on the associations of ozone exposure with cardiorespiratory function indices were separately explored. In addition, because childhood is a specific stage of growth and development, the results may not be generalized to broader populations.

Conclusions

In conclusion, indoor ozone exposure below the current indoor air quality limits was mainly associated with disorder in amino acid and bile acid metabolism. Ozone exposure was associated with the deteriorated cardiovascular function and FeNO by enhancing metabolism-related oxidation and inflammation. The study is valuable for improving the understanding of how indoor ozone at low concentrations affects children's cardiorespiratory function and the importance of strictly controlling indoor ozone pollution.

MATERIALS AND METHODS

Study Design and Participants

The repeated-measure panel study recruited 48 healthy children aged 11–14 years from a middle school in the suburban area of Beijing. Children with no health conditions, no history of thoracic surgery, who lived in Beijing for at least two consecutive years, and who stayed in the school dormitories on weekdays were eligible for the study. They were followed up for 1 week, and real-time exposure measurements including ozone were conducted inside the classrooms during weekdays throughout the study period. Health measurements and urine sample collection were conducted thrice in each participant. The participants were instructed to stay in the classrooms whenever possible and to record the time and place in the self-administered activity questionnaire if they stayed outdoors.

The study protocol was registered on *clinicaltrial.gov* (NCT03319056) and approved by the Institutional Review Board of Peking University Health Science Center.

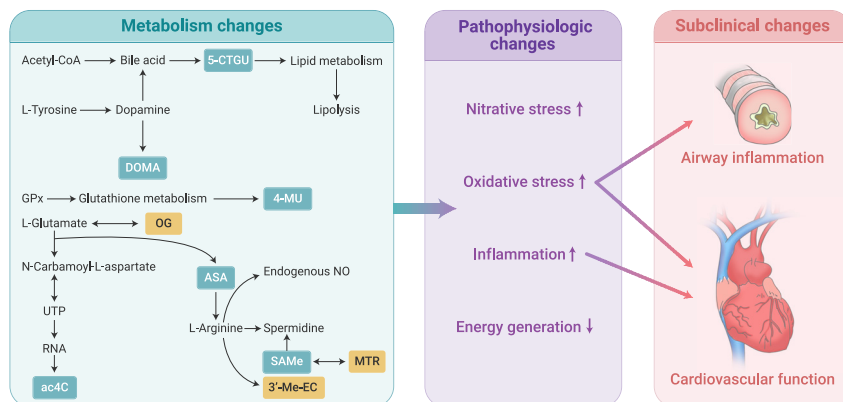


Figure 5. Schematic Diagram of the Hypothesized Mechanism that Links Indoor Ozone Exposure to the Observed Cardiorespiratory Effects. ■, Upregulation of Levels; ■, Downregulation of Levels. Abbreviations as in Figure 2.

A written informed consent was obtained from each participant and their guardians prior to the study enrollment.

Indoor Exposure Measurements

Indoor ozone, PM_{2.5}, PM₁₀, BC, noise, carbon dioxide (CO₂), and meteorological factors including temperature and relative humidity (RH) were monitored from 7:00 a.m. to 5:00 p.m. during weekdays, and were calculated within an average of 8 h (8:00 a.m. to 4:00 p.m.), which corresponded to the duration of cardiorespiratory measurements and urine collection. The related measurement devices were installed at the height of the breathing zone for the sitting children (about 1.2 m high from the floor) and were calibrated prior to the start of the study to ensure data quality. The instruments used to characterize indoor air pollution are described in the [Supplemental information](#).

Cardiorespiratory Function Measurements

The ambulatory electrocardiogram (ECG), BP, FeNO, and spirometry function measurements were conducted on each participant by trained investigators. The ECG variables monitored included HRV, HR, and ST-segment elevations (8:00 a.m. to 4:00 p.m.), and their 8-h averages were calculated for further analysis. BP, FeNO, and spirometry function, including FEV₁ and PEF, were measured at 5:00 p.m. Participants were instructed to refrain from performing exercises, consuming food (especially salty food), and drinking beverages that may affect their cardiorespiratory measurements. Details of the instruments used and procedures performed are provided in the [Supplemental information](#).

Urine Sampling and Metabolomics Analysis

The mid-stream specimens of the spot urine from each participant were collected at 5:00 p.m. on Monday, Wednesday, and Friday. The collected biospecimens were immediately transferred (at 4°C) to Peking University for processing and stored at -80°C prior to delivery to the laboratory of the Institute of Urban Environment, Chinese Academy of Sciences. The inter-laboratory transportation was conditioned with dry ice. Details of the sample preparation, UPLC-MS/MS data acquisition, and quality control are provided in the [Supplemental information](#). The processed mass feature tables were Pareto-scaled and introduced to the SIMCA-P software (v13.0, Umetrics, Uppsala, Sweden) for multivariate statistical analysis.

The metabolites were divided into three groups according to the tertiles of ozone exposure concentration (cut off: 8.7 ppb and 11.4 ppb) to explore the intergroup differences in metabolite relative abundance. A PLS-DA model was performed to determine the separation of urinary metabolite profiles at high, medium, and low ozone concentrations. In addition, a 999-time permutation test was used to check the model overfitting. The metabolites (i.e., variables) were screened using the PLS-DA model based on the following criteria: (1) the VIP value was >2, (2) the intensity difference of variables between high and low ozone concentration group was significant ($p < 0.05$), and (3) the association of ozone concentration with the log-transformed relative peak intensity of metabolite was significant ($q < 0.05$). The specific metabolite was identified by searching the HMDB (<https://hmdb.ca/>) using accurate mass measurements. Moreover, the UPLC-MS/MS product ion spectrum of a metabolite was matched with the MS spectra available in HMDB to confirm the identification. The web-based MetaboAnalyst software (<http://www.metaboanalyst.ca/>) and KEGG (Kyoto Encyclopedia of Genes and Genomes) database (<https://www.kegg.jp/>) were used for further metabolic pathway analysis. The flow chart of the MIMA analysis is shown in [Figure S7](#).

Statistical Analyses

Firstly, a trend test via linear mixed-effect model was applied to further explore the associations of ozone exposure with the subclinical indices of cardiorespiratory function. The low, medium, and high ozone concentration groups were classified according to the tertiles of ozone exposure concentrations with the lowest group as the reference, and the estimated percentage changes with the corresponding 95% confidence intervals of the cardiorespiratory function indices by tertiles of ozone were analyzed. Secondly, the Mann-Whitney nonparametric test was performed to evaluate the significance of intergroup differences of each metabolite. Thirdly, linear mixed-effect models were used to determine the significance of the associations of indoor ozone concentrations with the log-transformed relative peak intensity of each identified metabolite and to control for confounding variables. The models were adjusted for age, sex, BMI, class and long-term time trend (including day of measurement), BC, temperature, RH, and noise. In particular, BC was adjusted as it may interact with ozone on HRV/HR.⁸ The number of participants was included as a random effect. The collinear test results of these covariates are shown in [Table S5](#). Similar linear mixed-effect models were also utilized to explore the associations between ozone-related metabolites and cardiorespiratory function parameters. In order to explore the associations between ozone/health indices and metabolites in more detail, mixed-effect models were used for lag analysis. More information on the mixed-effect model and lag time definition can be found in the [Supplemental information](#). In addition, causal mediation analysis was conducted to strengthen the causal relationship with the same covariates, and an interaction analysis using a mixed-effect model was performed to

separately explore the interaction of sex and BMI with ozone concentrations on the metabolites.

Results were adjusted for multiple comparisons using the false discovery rate (FDR). Each FDR was estimated using q values; a q value of <0.05 was considered significant.^{13,14} Linear mixed-effect model and causal mediation analysis were conducted using the lme4, lmerTest, and mediation package of the R software (version 3.6.1; R Foundation for Statistical Computing). All statistical tests were two-sided with $\alpha = 0.05$. Moreover, ROC analysis was carried out to assess the specificity and sensitivity of the metabolites. The dependent variable was dichotomous, including the lowest (\leq first tertile) and highest ($>$ -third tertile) ozone concentration group divided by tertiles and metabolites were independent variables. Classic univariate ROC analysis and multivariate analysis of the combinational metabolite patterns were separately performed using SPSS 19 (SPSS Inc.) and online ROCCT (ROC Curve Explorer and Tester) software (<http://www.metaboanalyst.ca/>).

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AUTHOR CONTRIBUTIONS

F.D. and H.S. designed and supervised the study, interpreted the results, and revised the manuscript. S.L. and Q.H. analyzed the data, drafted and revised the manuscript. Q.H., X.Z., and B.N. were responsible for the acquisition of data on urinary metabolomics. F.D., W.D., W.Z., and D.Y. contributed to the ethical submission, recruitment of participants, exposure and health assessment. S.W., J.Z., and X.G. critically revised the manuscript and helped to interpret the data. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xinn.2021.100087>.

LEAD CONTACT WEBSITES

<http://sph.pku.edu.cn/info/1475/3855.htm>; <https://sph.xmu.edu.cn/2020/0423/c2690a400177/page.htm>.