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Research Paper

Metabolomic profiling reveals systemic metabolic disruptions induced by combined exposure to particulate matter and ozone

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ABSTRACT

Air pollution exposure, especially particulate matter (PM) and ozone (O_3), poses significant health risks, but the systemic metabolic consequences of combined exposures to PM and O_3, remain poorly understood. This study investigated systemic metabolic changes in male spontaneously hypertensive (SH) rats following inhalation exposure to concentrated ambient particles (CAPs) (PM2.5, 150 μg/m^3), ozone (O_3) (0.2 ppm), and their combination. Rats were exposed for 4 h, and serum samples were collected 1-hour post-exposure. Using targeted metabolomics, we identified significant alterations in metabolites involved in lipid metabolism (phosphatidylcholines), energy metabolism (acylcarnitine C3), and oxidative stress (glutamine). Notably, the combination exposure induced distinct metabolic changes, including increased acylcarnitine C3 levels, suggesting heightened mitochondrial dysfunction. Principal component analysis revealed overlapping profiles between CAPs and controls, indicating a subtler impact of CAPs compared to ozone or combined exposure. These systemic metabolic alterations are aligned with our previously published proteomics findings in cardiac tissues from the same rats, which showed elevated inflammatory markers (e.g., IL-6, TNF-α) and mitochondrial dysfunction. In conclusion, this study provides new insights into the systemic metabolic effects of air pollutant exposure, identifies novel metabolic targets of pollutant-induced toxicity, highlights the complex interactions resulting from combined exposure to multiple pollutants, and underscores the importance of assessing the combined effects of multiple pollutants in air pollution risk assessments.

Introduction

Air pollution, especially particulate matter (PM) and ozone (O_3), poses significant public health risks, contributing to a wide range of adverse outcomes, including cardiovascular and metabolic diseases (Miller et al., 2012; Jerrett et al., 2009). These pollutants induce oxidative stress, inflammation, and disruptions in metabolic pathways (Miller et al., 2012; Jerrett et al., 2009), which are increasingly implicated in the disease mechanisms. Both PM and O_3, are known to trigger metabolic alterations that are closely linked to their toxicological effects. Serum metabolites serve as systemic biomarkers, reflecting biological pathways disrupted by PM and O_3 exposure. (Nassan et al., 2021; Li, et al., 2017). Recent studies have highlighted PM's impacts on metabolic pathways. For instance, metabolomic profiling of plasma samples from the Normative Aging Study (Nassan et al., 2021) identified perturbed metabolites, such as glycerophospholipids, sphingolipids, and glutathione. These pathways are associated with inflammation, oxidative stress, immunity, and nucleic acid damage and repair – key processes linked to cardiovascular disease. Similarly, another study revealed associations between PM exposure and elevated biomarkers of oxidative stress, inflammation, insulin resistance, and altered blood pressure (Li, et al., 2017), highlighting the systemic health impacts of air pollution. Metabolomic studies have also linked O_3 exposure to cardiorespiratory dysfunction (Zeng et al., 2024; Cheng et al., 2024). O_3 exposure has been shown to disturb bile acid metabolism and nitric oxide signaling, which are critical for maintaining cardiovascular homeostasis (Zeng et al., 2024). In a metabolomic analysis of O_3 exposure, 43 metabolites were significantly altered, particularly those involved in the tricarboxylic acid (TCA) cycle, and amino acid metabolism, supporting the idea that ozone-induced metabolic disruptions may

Abbreviations: Gln, Glutamine; PC ae C40:1, Phosphatidylcholine acyl-alkyl C40:1; PC ae C42:3, Phosphatidylcholine acyl-alkyl C42:3; PC aa C24:0, Phosphatidylcholine diacyl C24:0; ACO2, Aconitase 2; LDHB, Lactate dehydrogenase-B.TNF-α, Tissue necrosis factor alpha; IL-6, Interleukin 6.

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contribute to cardiovascular disease (Cheng et al., 2024).

While the metabolomic impacts of PM and O_3 have been studied individually, the combined effects of these pollutants remain underexplored. Given that real-world air pollution exposure typically involves multiple pollutants, it is critical to investigate the interactive effects of co-exposure to PM and O_3. Understanding how these pollutants interact at the metabolic level can provide deeper insights into their – toxic effects, particularly on cardiovascular health. In a previous study, we evaluated the cardiac physiological effects of a single 4-hour inhalation exposure to summertime ambient PM (CAPs), O_3, or their combination in rats to explore their individual and interactive effects (Farraj et al., 2015). Exposure to CAPs and O_3, both individually and in combination, resulted in distinct effects on cardiac electrophysiology, myocardial vulnerability, and autonomic function (Farraj et al., 2015). Furthermore, proteomic profiling of cardiac tissue revealed differential inflammatory and metabolic responses (Ge et al., 2024). These results suggest that CAPs and O_3 likely engage multiple interacting pathways, which could exacerbate cardiovascular dysfunction.

This study builds on previous findings by utilizing serum metabolomics to evaluate systemic metabolic alterations in response to exposure to CAPs, O_3, and their combination. Key areas of investigation include assessing the impact of CAPs and O_3 on serum metabolites, identifying potential disruptions in specific metabolic pathways, and exploring how these metabolite changes relate to proteins changes identified from the same rats, with a focus on elevated inflammatory markers (e.g., IL-6, TNF-α). We hypothesized that co-exposure to CAPs and O_3 will induce more pronounced alterations in serum metabolites compared to individual exposures, reflecting disruptions in key pathways related to energy metabolism, oxidative stress, and inflammation.

Materials and methods

Materials

Concentrated ambient particles (CAPs) were generated from ambient air drawn into the exposure system, with a size-selective inlet that removed particulate matter (PM) greater than 2.5 μm, at the U.S. EPA's Research Triangle Park, NC, as previously described (Farraj et al., 2015). Ozone (O_3) was produced by passing compressed oxygen (O_2) through a silent arc ozone generator (OREC, Osmonics Corp.). For metabolomics analysis, we utilized the AbsoluteIDQ™ p180 metabolomics kit (Biocrates Life Sciences, Innsbruck, Austria). All solvents used in liquid chromatography-mass spectrometry (LC-MS) analyses were of chromatographic grade, and all other chemicals and solvents were of analytical grade. BCA protein assay kit for colorimetric detection and quantitation of total protein, were acquired from Thermo Fisher Scientific (Carlsbad, CA). IPA Software for identification of protein interaction network was a product from Qiagen (Redwood City, CA). The V-plex Proinflammatory Panel 2 kit for measurement of rat cytokine changes at expression levels in rat cardiac tissue was from Meso Scale Discovery (Gaithersburg, MD).

Animals

Twelve-week-old male SH rats (Charles River, Raleigh, NC) were housed in plastic cages (one per cage), maintained on a 12-hr light/dark cycle at approximately 22 °C and 50 % relative humidity in our Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, and held for a minimum of 1 week before telemeter implantation. This strain was chosen due to its genetic predisposition to hypertension, which enhances sensitivity to pollutant-induced cardiovascular effects. Male rats were selected to maintain consistency with prior research and to minimize variability associated with hormonal fluctuations in females. All rats had *ad libitum* access to food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and tap water. The same individual rats used in our previously published cardiac proteomics study (Ge et al., 2024) were utilized for this study. Serum samples were collected as part of the parallel proteomics study. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC). All animal experiments complied with the ARRIVE guidelines (Percie du Sert et al., 2020) and were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (NIH, 2011).

CAPs and ozone exposures

Particulate matter was concentrated using a Harvard Fine Particle Concentrator (HFPC, Harvard University, Boston, MA) which removed particles larger than 2.5 μm, as previously described (Farraj et al., 2015). The chemical composition of PM2.5 collected from the study site has been characterized in prior work (Aimen et al., 2015), showing it consists predominantly of organic carbon, elemental carbon, and trace metals (e.g., Fe, Zn, and Cu), alongside sulfates and nitrates. This composition is representative of urban ambient air pollutants in the study region and provides a basis for replicating this exposure scenario in future studies. All exposures were conducted during the morning hours (8:00 AM to 12:00 PM) to minimize variability due to circadian rhythms. Each exposure session lasted for 4 h, followed by sample collection within 1 h after the completion of the exposure. Air/PM mixtures were delivered into Hinners-style stainless steel and glass 0.3 m^3 whole-body chambers. Ozone was introduced into the chamber through the inlet duct, where it was mixed with the air/PM stream. Rats ($n = 6$/group) were exposed to three different scenarios: (i) filtered air as a control group, (ii) concentrated ambient particulate matter (CAPs, target concentration 150 $\mu g/m^3$), and (iii) CAPs combined with ozone (target concentration 0.2 ppm). This study design allowed for direct comparison of serum metabolomic results with previously published proteomics findings, while ensuring adherence to ethical principles. No additional animals were generated for this study. PM concentrations were monitored using gravimetric methods (Teflon filters, 47 mm, 2 μm pore, Pall Corp.) and real-time particle counting instruments, including a scanning mobility particle sizer (SMPS, TSI Inc.) and an aerodynamic particle sizer (APS, Model 3321, TSI Inc.), as described previously (Farraj et al., 2015).

Necropsy and serum sample collection

On the morning following the final exposure, rats were deeply anesthetized with an intraperitoneal injection of Euthasol (200 mg/kg sodium pentobarbital and 25 mg/kg phenytoin; Virbac Animal Health, Ft. Worth, Texas) (Farraj et al., 2015). Blood samples were collected via cardiac puncture, and serum was separated by centrifugation, snap-frozen in liquid nitrogen, and stored at −80 °C for metabolomics analysis.

Metabolomic profiling in serum

Serum samples were prepared in triplicate from each rat according to the manufacturer's protocol. Targeted metabolomics analysis of SH rat serum (10 μL) was performed using the AbsoluteIDQ™ p180 kit (Biocrates Life Sciences). Drying steps were performed using an Organomation Microvap 118 (Berlin, MA). The plate setup was randomized using Biocrates MetIDQ software (Beryllium version). The kit quantified a total of 186 metabolites (Supplemental Table 1), covering acylcarnitines, amino acids, hexoses, biogenic amines, glycerophospholipids, and sphingolipids. Quantification was performed via positive and negative electrospray ionization modes on an AB Sciex 4000 QTrap mass spectrometer (Framingham, MA) equipped with a Shimadzu LC system. Flow injection tandem mass spectrometry (FIA/MS/MS) was used to quantify lipids and hexoses, while reverse-phase liquid chromatography tandem mass spectrometry (LC/MS/MS) was employed for amino acids, biogenic amines, and acylcarnitines. Chromatographic separation was

achieved using an Agilent Zorbax Eclipse XDB-C18 column (2.1 × 30 mm, 3.5 μm) with 0.2 % formic acid in LC-MS grade water (Mobile Phase A) and 0.2 % formic acid in acetonitrile (Mobile Phase B). Quantification was achieved through multiple reaction monitoring (MRM) transitions and internal standards provided by the Biocrates kit.

Statistical analysis of metabolomics data

Metabolomics data were pre-processed and normalized before statistical analysis. All metabolite concentrations were log-transformed to stabilize variance and ensure normality. The normalized data were analyzed using the following steps. First, statistical comparisons between exposure groups (e.g., CAPs vs. CAPs + O_3, O_3 vs. CAPs + O_3) were conducted using a two-sample *t*-test for each metabolite. The *t*-test was used to evaluate whether the means of metabolite levels differed significantly between the two groups under study. For all statistical tests, a raw p-value of less than 0.05 was considered significant. Given the large number of metabolites analyzed, a False Discovery Rate (FDR) correction was applied to control for the possibility of false positives due to multiple testing. The FDR-adjusted p-values were calculated using the Benjamin-Hochberg procedure, which adjusts the raw p-values to account for multiple comparisons. Metabolites with an FDR-adjusted p-value less than 0.05 were considered statistically significant after correction. The fold change in metabolite levels was calculated as the ratio of the mean concentrations in the two groups. A fold change greater than 1 indicates an increase in the metabolite level in the treatment group, whereas a fold change less than 1 (or negative) indicates a decrease. In the present study, only metabolites with a relative fold change of ≥15 % (increase or decrease) and with p-values < 0.05 for both raw and FDR-adjusted values were considered significantly altered. Metabolites that passed these criteria were included in downstream pathway and functional analysis. Statistical analyses were performed using Python with the SciPy and stats models libraries, ensuring robust and reproducible analysis.

Additionally, Pearson correlation analysis was used to compute correlation coefficients for each pair of altered metabolite and cytokine variables (Ge et al., 2024). To establish statistically significant correlations, we employed criteria: correlation coefficients exceeding 0.6 with a significance level of $p < 0.05$.

Results

Overview of serum metabolomic changes across all treatments and comparisons

Metabolite alterations in the serum of rats exposed to CAPs and O_3 were identified by comparing the metabolic profiles of four experimental groups: rats exposed to filtered air (control), CAPs, O_3, and CAPs + O_3 (exposure groups). Dynamic changes in serum metabolites were observed following exposure CAPs, O_3 and their combination. Phosphatidylcholines (PCs) followed similar trends across all groups, with concentrations rising progressively from CAPs to CAPs + O_3 to O_3, while the CAPs group displayed slightly lower levels than the air control group (Fig. 1A), suggesting a potential compensatory response. This phenomenon warrants further investigation to elucidate whether it represents homeostatic recovery or another underlying mechanism. In addition to PCs, several amino acids, such as kynurenine and putrescine, displayed notable fold changes compared to the air control group (Fig. 1B). Amino acids generally showed higher concentrations in the CAPs + O_3 group than in the CAPs or O_3 alone groups, although most were downregulated compared to air. A significant finding was the marked increase in Acylcarnitine C3 in the CAPs + O_3 group compared to the other groups (Fig. 1C), suggesting a disruption in fatty acid oxidation. Acylcarnitine C3 is a key intermediate in mitochondrial energy metabolism (Makrecka-Kuka et al., 2017) and its accumulation indicates impaired β-oxidation and mitochondrial dysfunction under combined CAPs and ozone exposure.

The statistical significance of the metabolite changes observed across the experimental groups varied. Some metabolites did not show significant alterations based on raw and FDR-adjusted p-values (Fig. 1A), while others were significant in raw p-values but did not pass the more stringent threshold for FDR-adjusted p-values (Fig. 1B). The metabolites that showed significant changes based on raw p-values but were not significant after FDR p-values adjustment are listed in Supplemental Table 2. Although co-exposure resulted in notable metabolic changes (Supplemental Table 2), the trends in metabolite levels suggest a complex interaction between CAPs and O_3 exposures, where the combination may invoke compensatory or antagonistic mechanisms compared to individual exposures. In this study, only the metabolites that were statistically significant in both raw and FDR-adjusted p-values were considered to represent true and meaningful changes (Fig. 1C). This approach ensures that the findings reported here are robust and reliable, minimizing the risk of false positives and providing greater confidence in the biological relevance of the observed metabolic alterations. Table 1 lists the altered metabolites that were significant in both raw and FDR-adjusted p-values, and these metabolites were selected for further pathway and functional analysis. To provide a clearer representation of these changes, we have included graphical representations (Fig. 2) showing the average and standard error for each metabolite.

A supervised partial least squares discriminant analysis (sPLS-DA) for all treatments demonstrated that the air and CAPs groups clustered closely together, suggesting high similarity. In contrast, CAPs + O_3 and O_3 clustered separately from the air and CAPs groups, indicating significant differences between these exposures (Fig. 3) and the unique metabolic signatures associated with these exposures.

Alterations of phosphatidylcholines (PCs) in serum: CAPs vs. O_3

In the comparison between CAPs and O_3, three phosphatidylcholines (PCs) exhibited significant changes: PC ae C40:1, PC ae C42:3, and PC aa C24:0. PC aa C24:0 was increased by 2.44-fold, while PC ae C40:1 and PC ae C42:3 was decreased by 0.61-fold and 0.43-fold, respectively (Table 1). The significant increase in PC aa C24:0 in the CAPs group compared to O_3 suggests that certain lipid species may be upregulated by particulate matter exposure, while the significant decreases in PC ae C40:1 and PC ae C42:3 in the CAPs group compared to O_3 indicate that CAPs may reduce these specific phosphatidylcholines.

Serum metabolite alterations: CAPs vs. CAPs + O_3

In the comparison between CAPs exposure alone and the combined exposure of CAPs and O_3, levels of Acylcarnitine C3 and Gln were significantly increased in the CAPs + O_3 group compared to the CAPs group. Compared to the CAPs + O_3 group, Acylcarnitine C3 and Gln in CAPs alone was decreased with a fold change of 0.34 and 0.28, respectively (Table 1).

Serum metabolite alterations: O_3 vs. CAPs + O_3

In the comparison between ozone (O_3) exposure alone and the combined exposure of CAPs and O_3, levels of Acylcarnitine C3 were significantly increased in the CAPs + O_3 group compared to the O_3 group (Table 1). Additionally, phosphatidylcholine species were notably altered. PC aa C24:0, a saturated phosphatidylcholine important for maintaining membrane stability and structural integrity (González-Ramírez et al., 2020; Van der Veen et al., 2017), showed a marked reduction in the CAPs + O_3 group compared to O_3 alone (Fig. 4). Ether-linked phosphatidylcholines were also affected. PC ae C40:1, which plays a role in cell signaling, membrane fluidity, and antioxidative functions (Engelmann, 2004), exhibited a reduction in the CAPs + O_3 group compared to O_3 alone, with a fold change of 0.84 (Table 1). This reduction may indicate an increase in oxidative stress in the combined

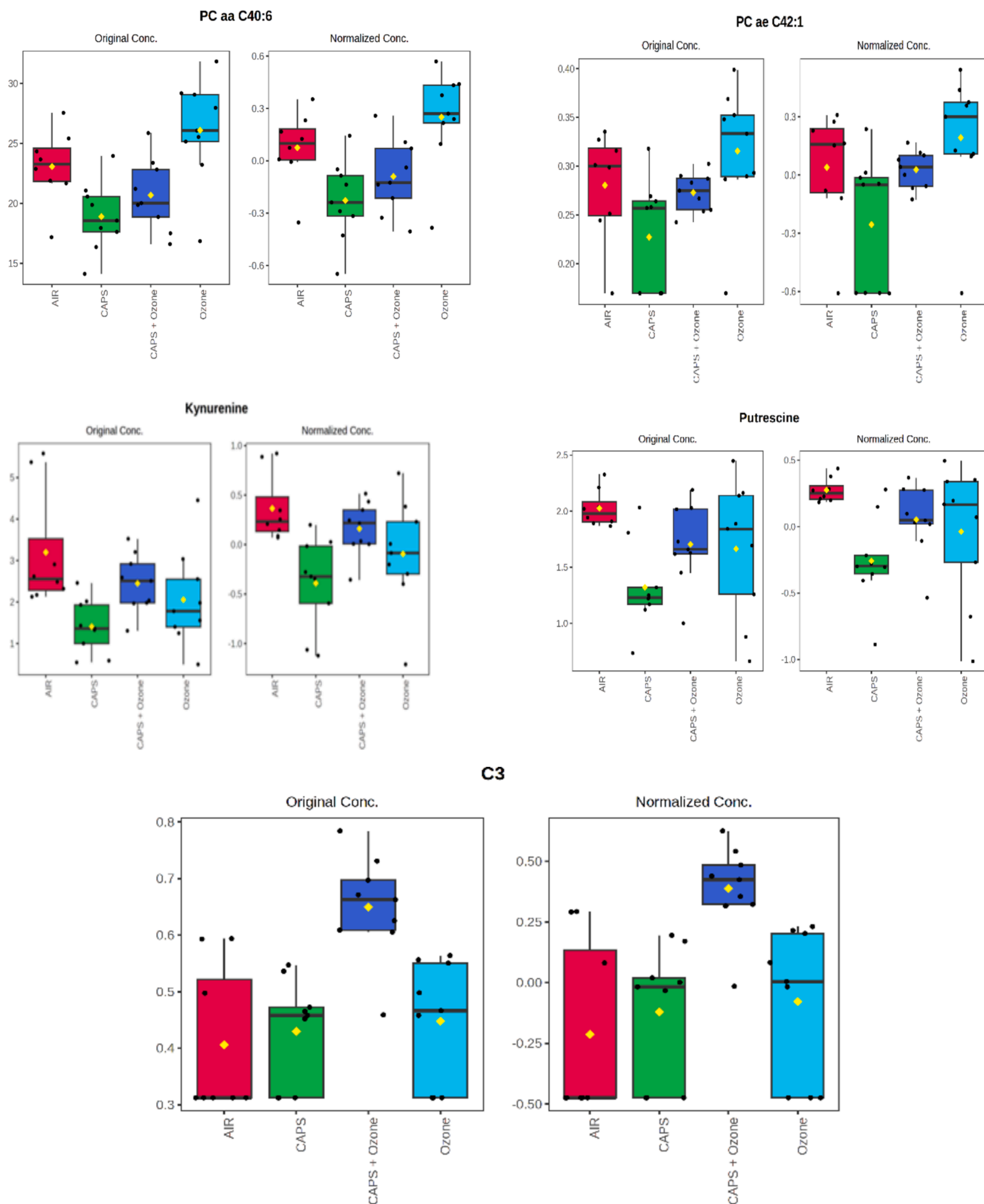

Fig. 1. Serum metabolite changes in response to Air, CAPs, O_3 , and CAPs + O_3 exposure. (A) Phosphatidylcholines (PCs) exhibited differential expression across the exposure groups.(B) Amino acid changes, including kynurenine and putrescine, are shown.(C) Acylcarnitine C3 showed a marked increase in the CAPs + O_3 group compared to other exposure groups, indicating impaired fatty acid oxidation. Panels (A), (B), and (C) provide side-by-side comparisons of Mean (+/-SE) metabolite levels across exposure groups. Some metabolite changes in the co-exposure group appear similar to control values. Observed differences are based on specific statistical significance thresholds.

Table 1
List of metabolites with significant fold changes for CAPs vs. Ozone, CAPs vs. CAPs + Ozone, and Ozone vs. CAPs + Ozone. Both raw p-values and FDR-adjusted p-values are presented. Only metabolites with significant changes in both raw and FDR-adjusted p-values ($p < 0.05$) are included, ensuring that the results account for the multiple comparisons made across the metabolites tested.

Metabolite	Comparison	Fold change (± SE)	Raw p-value	FDR-adjusted p-value
PC ae C40:1	CAPs vs. Ozone	0.61 ± 0.02374	0.0042	0.039
PC ae C42:3	CAPs vs. Ozone	0.43 ± 0.03121	0.0026	0.043
PC aa C24:0	CAPs vs. Ozone	2.44 ± 0.06213	0.0075	0.043
Acylcarnitine C3	CAPs vs. CAPs + Ozone	0.34 ± 0.0583	0.0018	0.032
Gln	CAPs vs. CAPs + Ozone	0.28 ± 0.0217	0.0281	0.047
Acylcarnitine C3	Ozone vs. CAPs + Ozone	0.40 ± 0.0675	0.00123	0.029
PC aa C24:0	Ozone vs. CAPs + Ozone	0.11 ± 0.0182	0.027	0.04
PC ae C40:1	Ozone vs. CAPs + Ozone	0.84 ± 0.1204	0.0085	0.038
PC ae C42:3	Ozone vs. CAPs + Ozone	0.56 ± 0.0783	0.0089	0.045

Significant changes are defined based on raw p-values (<0.05) and FDR-adjusted p-values (<0.05) to account for multiple comparisons.

exposure group. Similarly, PC ae C42:3, another ether-linked phosphatidylcholine involved in antioxidative processes and membrane dynamics (Furse and de Kroon, 2015 was reduced by a fold change of 0.56 in the CAPs + O_3 group compared to O_3 alone (Table 1). These reductions in phosphatidylcholines suggest an imbalance in cellular redox homeostasis and a potential compromise of membrane integrity when particulate matter and ozone exposures are combined. Overall, these findings suggest that co-exposure to CAPs and ozone influences metabolic pathways distinct from individual exposures, potentially reflecting compensatory or antagonistic interactions rather than additive effects.

Pearson correlation analysis of altered cytokines and serum metabolites

Using Pearson correlation analysis, we conducted a quantitative assessment of the relationships between the altered cytokines that were identified from previous proteomic study (Ge et al., 2024) and serum metabolites in the same rats exposed to CAPs and ozone by calculating correlation coefficients (Table 2). Pearson correlation coefficients quantify the degree of association between cytokines and metabolites. Higher coefficients indicate stronger associations, suggesting that these cytokines and metabolites may exhibit similar patterns of change. As depicted in Table 2, IL-6 was positively correlated with Acylcarnitine C3 ($r = 0.6$, $p = 0.015$) and Gln ($r = 0.68$, $p = 0.003$) in the CAPs vs. CAPs + Ozone comparison. In the Ozone vs. CAPs + Ozone comparison, IL-6 was negatively correlated with phosphatidylcholines, including PC aa C24:0 ($r = -0.64$, $p = 0.004$), PC ae C40:1 ($r = -0.61$, $p = 0.007$), and PC ae C42:3 ($r = -0.6$, $p = 0.008$). For TNF-α, we observed positive correlations with phosphatidylcholines in the Ozone vs. CAPs + Ozone comparison, including PC aa C24:0 ($r = 0.6$, $p = 0.017$), PC ae C40:1 ($r = 0.65$, $p = 0.04$), and PC ae C42:3 ($r = 0.71$, $p = 0.008$).

Discussion

This study investigated the systemic metabolic disruptions induced by combined exposure to particulate matter (PM) and ozone (O_3) in a rodent model. Key findings include significant alterations in phosphatidylcholines, acylcarnitine C3, and glutamine, which highlight disrupted pathways in lipid and energy metabolism and oxidative stress. The co-exposure to PM and O_3 resulted in more pronounced metabolic changes compared to individual exposures. These findings were further supported by correlations between altered serum metabolites and previously identified cardiac proteomic markers, providing insights into the interplay between systemic and tissue-specific effects of air pollution. The implications of these results for understanding pollutant-induced cardiovascular risks and their relevance to environmental health risk assessments are discussed.

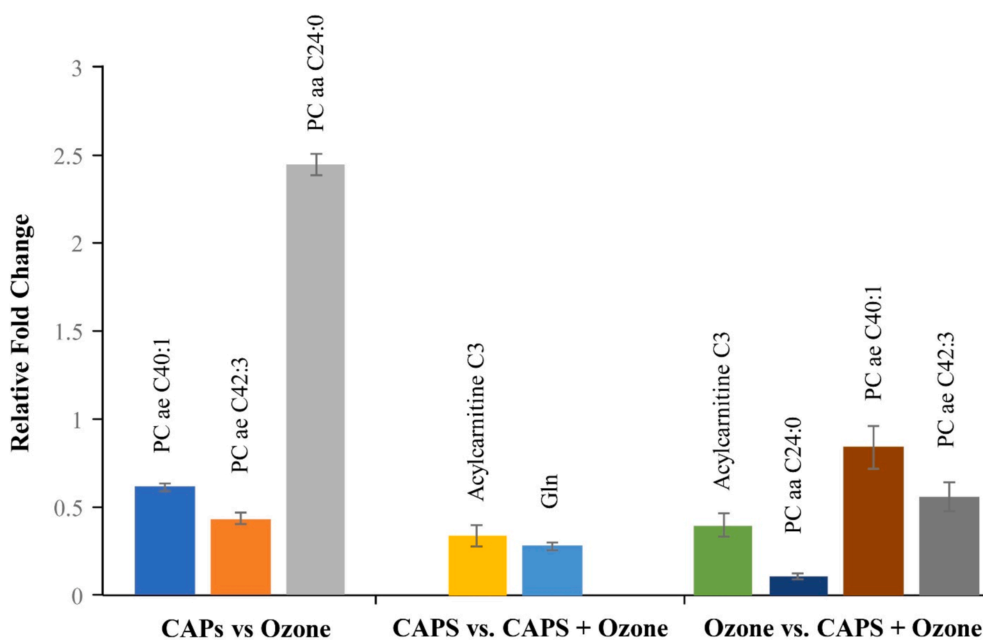

Fig. 2. Graphical representation of metabolites with significant fold changes for CAPs vs. Ozone, CAPs vs. CAPs + Ozone, and Ozone vs. CAPs + Ozone. Only metabolites with significant changes in both raw and FDR-adjusted p-values ($p < 0.05$) are included. Data are presented as relative fold change ± standard error.

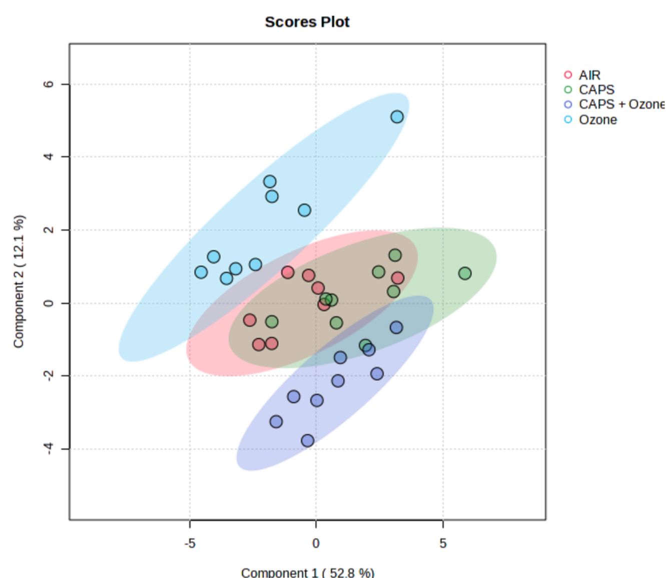

Fig. 3. Partial least squares discriminant analysis (sPLS-DA) of metabolite profiles across exposure groups. The sPLS-DA plot demonstrates clustering of air and CAPS exposure groups, while the CAPs + O_3 and O_3 groups cluster separately, indicating distinct metabolomic profiles following co-exposure. The overlap between CAPs and control groups in the sPLS-DA plot highlights variability in individual responses to exposures, while co-exposure groups show distinct clustering.

Key metabolic pathways and implications of co-exposure to CAPs and ozone

Lipid metabolism, cellular membrane integrity, and oxidative stress

Changes in lipid metabolites, particularly phosphatidylcholines (PCs), indicate substantial disruptions in lipid metabolism and membrane stability. PCs are essential components of cell membranes, playing key roles in maintaining fluidity, structural integrity, and cell signaling (Horn and Jaiswal, 2019). The increase in PC aa C24:0 in the CAPs and CAPs + O_3 groups suggests particulate matter exposure promotes lipid accumulation and alters membrane composition. This likely reflects a cellular response to oxidative stress, where cells attempt to stabilize their membranes under adverse conditions (Rao et al., 2018). In contrast, the elevated levels of ether-linked phosphatidylcholines (e.g., PC ae C40:1 and PC ae C42:3) in the co-exposure group suggest a disruption in antioxidative defense mechanisms. Ether-linked PCs, such as PC ae C40:1 and PC ae C42:3, are involved in protecting cells from oxidative damage and their elevation indicates heightened oxidative stress (Chen et al., 2023). This aligns with the growing body of evidence that oxidative stress is a central mechanism through which air pollutants induce inflammation and cellular dysfunction (Lodovici and Bigagli, 2011). The increased lipid alterations in the CAPs + O_3 group further suggest that co-exposure exacerbates oxidative stress, leading to more pronounced lipid peroxidation and membrane destabilization compared to individual pollutant exposures (Rainey et al., 2017).

Altered amino acid metabolism

Glutamine (Gln), an amino acid essential for protein synthesis, nitrogen metabolism, and cellular stress responses (Goswami et al., 2024) was significantly elevated in the CAPs + O_3 group, but not with exposure to each alone. This elevation suggests increased metabolic stress on nitrogen metabolism and reflects the body's attempt to bolster protein synthesis and repair mechanisms and reflects the body's attempt to bolster protein synthesis and repair mechanisms in response to oxidative and inflammatory damage (Masenga et al., 2023). The proteomics data in the heart tissue in our previous manuscript, which show elevated levels of inflammatory cytokines such as IL-6 and TNF-α [8], provide further support for this hypothesis. These cytokines are known to influence amino acid metabolism and may have contributed to the elevation of glutamine in response to environmental pollutants (Dos Santos Quaresma et al., 2020).

Fatty acid oxidation and mitochondrial dysfunction

The elevation of Acylcarnitine C3, a marker of disrupted fatty acid oxidation, in the CAPs + O_3 group suggests that combined exposure leads to mitochondrial dysfunction and incomplete fatty acid oxidation (Gander et al., 2021). This observation is consistent with previous

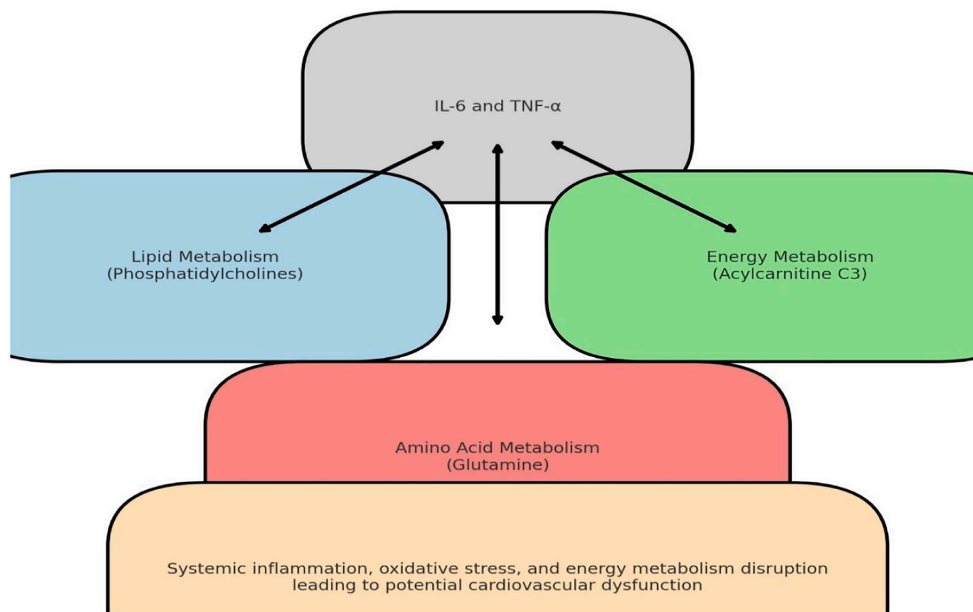

Fig. 4. Proposed conceptual pathway linking serum metabolomics and cardiac tissue proteomics. This diagram depicts the proposed mechanistic links between serum metabolite changes and cardiac tissue proteomic findings. Metabolites such as Acylcarnitine C3 and phosphatidylcholines are shown as part of broader metabolic pathways that interact with proteins identified in the cardiac tissue proteomics analysis. The arrows indicate the direction of these interactions, and the boxes represent key components of the pathways.

Table 2
Pearson correlations of the differentially expressed cytokines and metabolites in rats exposed to CAPs and ozone.

	CAPs vs CAPs + Ozone		Ozone vs CAPs + Ozone			
	IL-6		IL-6		TNF-α	
	Coefficient	P value	Coefficient	P value	Coefficient	P value
Acylcarnitine C3	0.6	0.015				
Gln	0.68	0.003				
PC aa C24:0			−0.64	0.004	0.6	0.017
PC ae C40:1			−0.61	0.007	0.65	0.04
PC ae C42:3			−0.6	0.008	0.71	0.008

studies showing that air pollutants interfere with mitochondrial function, reduce ATP production, and increase reactive oxygen species (ROS) generation (Reddam et al., 2022). The increased Acylcarnitine C3 levels observed here may indicate a greater metabolic burden on mitochondrial function under combined exposure, which may contribute to oxidative stress and systemic inflammation.

CAPs and O_3 exposure reveals distinct patterns of metabolic disruption

The metabolic disruptions observed in this study provide insight into the distinct physiological impacts of CAPs and O_3 exposure. The reductions in ether-linked phosphatidylcholines (PC ae C40:1 and PC ae C42:3) in the CAPs exposure group highlight the oxidative stress induced by PM. However, the CAPs + O_3 group exhibited levels comparable to controls, potentially indicating compensatory metabolic adjustments during co-exposure. (CAPs vs O_3, Table 1). The significant reduction in these PCs suggests that CAPs exposure may overwhelm cellular defense mechanisms, leading to compromised membrane stability, especially in oxidative-stress-sensitive tissues such as the heart (Gangwar et al., 2020). This is consistent with research linking PM exposure to increased ROS generation, inflammation, and chronic cardiovascular and respiratory disease (Lim and Kim, 2024). Conversely, the increase in PC aa C24:0 in the CAPs group suggests a compensatory cellular response. PC aa C24:0 is a fully saturated phosphatidylcholine known for enhancing membrane stability (Rao et al., 2018). Its upregulation may represent an adaptive response to oxidative damage by stabilizing cell membranes through the incorporation of less oxidizable lipids. This dynamic adjustment in lipid metabolism underscores the complexity of the body's response to environmental stressors.

In contrast, O_3 exposure had a less pronounced effect on these specific phosphatidylcholines (O_3 vs CAPs + O_3, Table 1), suggesting that the mechanisms of toxicity may differ between O_3 and PM. While O_3 exposure induces oxidative stress primarily in the respiratory system (Wiegman et al., 2020), CAPs seem to exert more systemic effects, at the respective pollutant concentrations used in the present study. These results suggest that while both pollutants disrupt lipid metabolism, the mechanisms differ, with CAPs having a broader impact on systemic lipid homeostasis.

Interaction effects of co-exposure to CAPs and O_3

The metabolomic profiles of the CAPs + O_3 group clustered distinctly from those of the CAPs and O_3 groups in multivariate analysis, highlighting the unique metabolic signature induced by co-exposure. This suggests that combined CAPs and O_3 exposure results in non-linear interactions that exacerbate toxicity, rather than simply additive effects. The significant increase in Acylcarnitine C3 in the CAPs + O_3 group reflects disrupted fatty acid oxidation, potentially due to the compounded effects of oxidative stress and inflammation during co-exposure. This trend, despite statistical considerations, underscores the potential for interaction effects between CAPs and O_3. Acylcarnitine C3, a critical marker of mitochondrial fatty acid oxidation, was elevated in the co-exposure group, when compared to CAPs or O_3 alone. These results indicate that co-exposure results in greater disruption of mitochondrial function and energy metabolism than either pollutant alone. The accumulation of Acylcarnitine C3 suggests impaired β-oxidation, potentially due to oxidative stress or inflammation, leading to mitochondrial dysfunction. Given that energy production is crucial for high-demand tissues such as the heart, these metabolic disruptions could contribute to the heightened cardiovascular risk associated with air pollution exposure. However, it is critical to interpret these findings cautiously. While the distinct clustering and Acylcarnitine C3 changes suggest interactive effects, the study design was not specifically intended to evaluate synergistic toxicity. The absence of multiple dose groups and a broader exploration of interaction models limits the capacity to definitively classify the observed effects as synergistic. Instead, these findings highlight potential interaction effects that warrant further investigation in more robust experimental frameworks designed explicitly for synergy assessment.

Although co-exposure resulted in notable metabolic changes (Supplemental Table 2), these differences did not reach statistical significance compared to air when using FDR-adjusted p-values. This may be due to the stringent nature of the correction method or the body's capacity to maintain homeostasis under baseline conditions. Additionally, this study focused on a specific subset of metabolites in serum, which may not fully capture the complexity of biological responses to CAPs and/or O_3 exposure. Furthermore, only a limited number of biological replicates were examined. While using a modest number of biological replicates is standard in metabolomics research, increasing the number of replicates could have enhanced the robustness of our findings.

Correlation between proteomics and metabolomics findings

Our previous proteomics findings revealed significant changes in inflammatory cytokines of IL-6 and TNF-α, both of which are central mediators of oxidative stress, inflammatory and metabolic disruptions (Ge et al., 2024). The role of IL-6 and TNF-α extends beyond inflammatory signaling, as these cytokines also regulate lipid metabolism [Górecka et al., 2022; Kern et al., 2018). For IL-6, significant positive correlations were found with Acylcarnitine C3 and Gln in the CAPs vs. CAPs + O_3 comparison. This suggests that heightened inflammation, indicated by increased IL-6 levels, is closely associated with disruptions in energy metabolism and nitrogen balance, particularly reflecting mitochondrial dysfunction and a stress response to pollutant exposure. In the O_3 vs. CAPs + O_3 comparison, IL-6 was negatively correlated with phosphatidylcholines, which suggest that co-exposure to CAPs and O_3 exacerbates oxidative stress, leading to lipid depletion and compromised membrane integrity. TNF-α, another key inflammatory marker, is associated with increased lipid levels, possibly indicating a protective cellular response aimed at preserving membrane stability under oxidative stress conditions. In summary, these correlations reveal a complex interaction between inflammation and metabolic disruption in response to pollutant exposure.

The overlap between proteomics and metabolomics findings is also evident in the disruptions of energy metabolism. The proteomics data indicated significant alterations in proteins involved in mitochondrial function, such as Aconitase 2 (ACO2), a key enzyme in the TCA cycle,

and Lactate dehydrogenase-B (LDHB), involved in lactate metabolism (Ge et al., 2024). These changes suggest impaired mitochondrial energy production, which is supported by the metabolomics findings. The significant increase in Acylcarnitine C3, a marker of disrupted fatty acid oxidation, aligns with the proteomics data, and indicates that mitochondrial dysfunction is a key consequence of CAPs + O_3 exposure. This impaired energy metabolism is likely exacerbated by the oxidative stress and inflammatory responses. The co-exposure to CAPs and ozone elicited more pronounced metabolic disruptions compared to either pollutant alone. The downregulation of mitochondrial proteins in the heart correlates with the increase in serum Acylcarnitine C3 (Dambrova et al., 2022), reflecting a systemic disruption in energy metabolism. We propose the following conceptual pathway (Fig. 4) illustrating the interactions between the observed metabolic and proteomic changes to provide a framework for understanding the broader health implications of CAPs and O_3 exposure.

Conclusion

This study is the first to integrate serum metabolomics and tissue proteomics to increase understanding of the systemic and tissue-specific effects of co-exposure to CAPs and O_3. The combination of the metabolomics data in serum with our previously published proteomics data from cardiac tissue highlight the complex biological pathways affected by air pollution and underscores the importance of addressing the combined effects of multiple pollutants in future health risk assessments. These findings also provide insights into the mechanisms underlying cardiovascular and metabolic diseases associated with environmental exposures. The extent to which these metabolic alterations influenced our previously reported physiological effects is unclear and requires further mechanistic investigation. Nevertheless, the evidence for metabolic disruption and the previous linkage of metabolic factors with adverse health effects suggests that such factors, perhaps both in the short term and with repeated exposure over time, may serve as potential biomarkers for pollutant-induced toxicity.

CRediT authorship contribution statement

Yue Ge: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Supervision, Visualization, Writing – original draft. **Maliha S. Nash:** Methodology, Formal analysis, Data curation, Visualization. **Aimen K. Farraj:** Methodology, Investigation, Data curation, Writing – review & editing.

Declaration of competing interest

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

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Disclaimer

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2025.100216.

Data availability

Data will be made available on request.

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