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Plasma Proteomic Associations with Short-, Intermediate-, and Long-Term Ozone Exposure in a Han Chinese population

Kaixuan Li,[▽] Wenran Li,[▽] Yue Niu,[▽] Xiyang Cai, Xia Meng, Li Jin, Haidong Kan,* and Sijia Wang*

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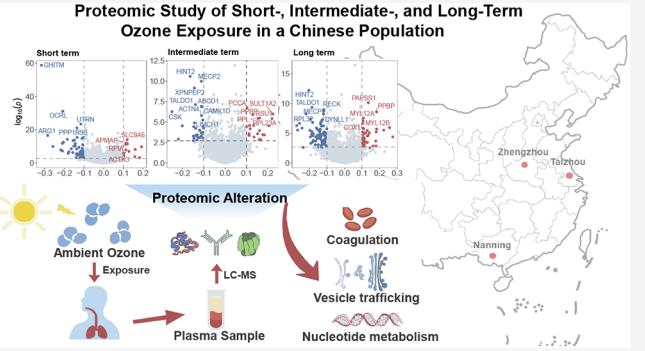
ABSTRACT: While epidemiological evidence consistently links ambient ozone to higher rates of disease and death, the biological mechanism and processes driving this association remain unclear. In this study, we recruited 2623 Han Chinese participants and measured the expression of 6528 plasma proteins using untargeted high-resolution mass-spectrometry-based proteomics. Individual-level ozone exposure was assessed at short-term, intermediate-term, and long-term durations. Proteome-wide association analysis identified 209 proteins significantly associated with ozone exposure across three exposure windows, including 74 proteins for short-term, 52 for intermediate-term, and 125 for long-term. Moreover, comparative analyses of proteins associated with three exposures revealed that longer ozone exposure magnifies proteomic effects. The ozone-related proteins were significantly enriched in biological pathways related to coagulation, vesicle trafficking, and nucleotide metabolism and have previously been linked to a variety of diseases, such as cardiovascular, respiratory, immune, and metabolic diseases. Collectively, our findings offer a proteome-wide perspective on the biological mechanisms underlying ozone toxicity, supported by evidence from a Chinese cohort.



KEYWORDS: *ozone, proteomics, biological pathways, cohort study*

1. INTRODUCTION

As the world's largest single risk factor among all environmental and lifestyle hazards, air pollution is responsible for an estimated 9 million deaths annually, accounting for 16% of all fatalities globally.[1,2] In recent years, the health threats posed by ozone-related mortality and morbidity have drawn increasing attention.[3] Clinical observations have consistently demonstrated connections between ozone pollutant exposure and elevated risks for cardiovascular diseases, including stroke, ischemic heart disease, heart failure, and blood vessel blockage.[4−6] Despite these epidemiological findings, the biological mechanism and causal pathways linking ozone exposure to disease outcomes remain to be fully delineated at the molecular level.

Research investigations focusing on population health have identified associated biomarkers in inflammatory signals and blood vessel wall function under ozone exposure.[7−9] Representative examples include soluble P-selectin indicating platelet activation; fractional exhaled nitric oxide and the sum of nitrite and nitrate in exhaled breath condensate reflecting airway inflammation; endothelin-1 and angiotensin-converting enzyme indicating vasoconstrictive endothelial signaling; 8-isoprostane reflecting systemic oxidative stress.[10−13] However, the use of hypothesis-driven biomarker selection has limited a comprehensive understanding of ozone-induced molecular changes. Moreover, previous studies focused on the short-term ozone exposure, and there remains a critical knowledge gap in mechanistic studies investigating monthly and annual exposure time durations in a real-world environment.

Advancements in proteomics technologies in recent years have significantly enhanced the ability of contemporary analytical platforms to perform comprehensive molecular screening. It is now possible to investigate the molecular pathways linking air pollution and health in a nonhypothesis-driven manner.[14−18] Nevertheless, the majority of existing studies have focused on Caucasian populations in Europe, yet large, well-characterized cohorts in Chinese populations are scarce—despite China's distinct and rising O_3 exposures and potentially underestimated cardiopulmonary burden—highlighting the urgent need for evidence specific to Chinese populations.[19]

Therefore, to fill the existing research gap, we established the first large-scale proteome-wide association study of ambient

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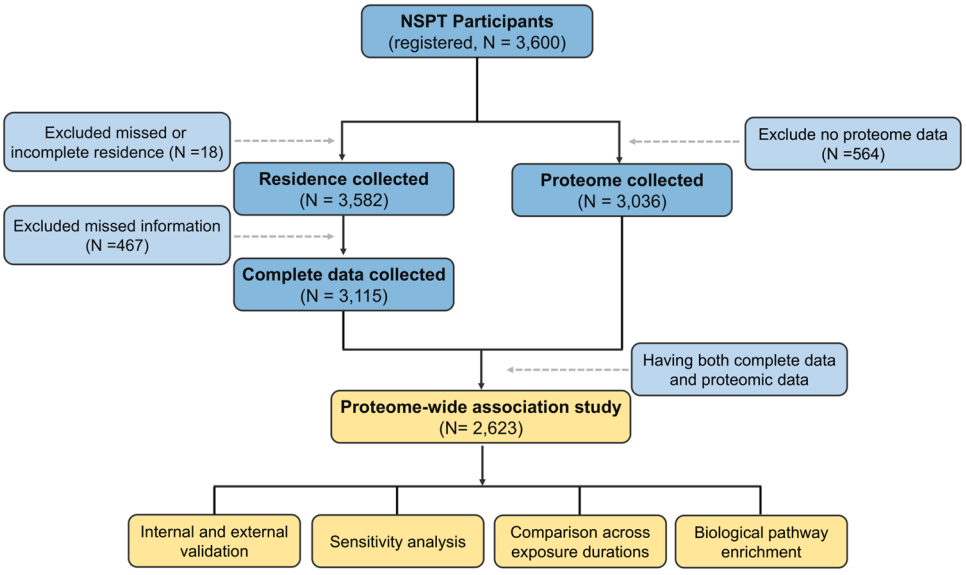

Figure 1. Flowchart of individuals in this study. Among 3600 registered NSPT participants, 18 individuals with missing or incomplete residence information and 564 without proteomic data were excluded. After further excluding 467 participants with missing covariate information, a total of 2623 participants with both complete exposure and proteomic data were included in the final proteome-wide association study. Subsequent analyses included internal and external validation, sensitivity analyses, comparisons across exposure durations, and biological pathway enrichment.

ozone exposure in a Chinese cohort to elucidate the proteomic associations with ozone exposure. Ambient ozone exposure was categorized into short-term (1 month), intermediate-term (1 year), and long-term (10 years), and separate proteome-wide association analyses were conducted for each duration. We compared the effects of different exposure durations on associated proteins and performed biological enrichment analyses to reveal the potential mechanisms and pathways underlying the impact of ozone exposure on human health.

2. MATERIALS AND METHODS

2.1. Study Population

The National Survey of Physical Traits (NSPT) cohort study was conducted as a population-based investigation encompassing participants of Chinese nationality. The study implemented a multistage sampling approach across diverse suburban regions of China. Data collection occurred at four distinct time points: August 2015 in Taizhou, Jiangsu; July 2017 in Zhengzhou, Henan; March 2018 in Nanning, Guangxi; March 2019 in Taizhou, Jiangsu. These subgroups, representing northern, southern, and eastern China, respectively, capture both regional characteristics and population heterogeneity within the Chinese population. Subject recruitment employed a randomized selection process to enroll volunteers with no selective inclusion criteria applied, and individuals with critical diseases were excluded. Blood samples were collected concurrently at each site during the recruitment period. At the time of enrollment, participants' residential street addresses were obtained through a standardized questionnaire (Table S1), and these geocoded data were subsequently utilized for ozone exposure prediction. The detailed workflow of the cohort and study design is presented in Figure 1.

The study incorporated a set of covariates, all of which were collected through a standardized personal questionnaire. These covariates included demographic factors (sex and age), lifestyle variables (smoking status, smoke pack years, passive smoking exposure, and alcohol consumption), and socioeconomic indicators (education level and household income). Body mass index (BMI) was computed using height and weight data from on-site physical exams. To adjust for potential population structure, we used principal components (PCs) based on genotype data in our models.

This study was approved by the Ethics Committees of Fudan University (14,117) and the Shanghai Institutes for Biological Sciences (ER-SIBS-261410), and all participants provided written informed consents.

2.2. Ozone Exposure Assessment

A comprehensive, high-resolution data set of maximum daily 8-h average (MDA8) ozone levels was generated for mainland China, spanning the years 2005 to 2019. The predictions, which offer full spatiotemporal coverage, were produced at a 1 km × 1 km spatial and daily temporal resolution using a random forest algorithm. The methodological details and validation process have been previously published.[20] The random forest models were developed with ground-level MDA8 ozone measurements as the response variable. The models were trained by using a set of predictors that included ozone simulations from the Community Multiscale Air Quality (CMAQ) model, meteorological parameters, population density, elevation, and road network data. The model performance was evaluated through cross-validation, yielding an overall R^2 of 0.80 and a root-mean-square error (RMSE) of 20.93 $\mu g/m^3$ when comparing modeled and observed daily ozone concentrations. Before 2013, China had not yet initiated the routine monitoring of environmental ozone exposure. We necessarily relied on model-predicted exposures for those earlier periods, which is common practice in environmental epidemiology.[21,22] The monthly and annual mean ozone concentrations were calculated for each 1 km grid cell, providing a comprehensive data set of ozone exposure at various temporal scales.

We calculated the monthly and yearly mean ozone concentrations for each participant based on their residential information and the corresponding grid-based exposure data. From this time series, we defined three distinct exposure windows relative to each participant's blood sampling date. Short-term exposure was quantified as the mean concentration in the month of sampling; intermediate-term exposure represented the mean over the preceding 12 months; long-term exposure was characterized as the average ozone concentration from May to October for each year during the 10 year period preceding each participant's blood draw date. We used the warm-season average instead of the annual mean because the World Health Organization (WHO) Air Quality Guidelines recommend using warm-season average concentrations to define long-term exposure, and averaging over 10 years may underestimate the health impacts of high ozone levels.[5,23−26] Each of these exposure windows (short-term, intermediate-term, and long-term) was then analyzed separately.

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Table 1. Summary of Participant Characteristics in the NSPT Cohort

	total (N = 2623)	Taizhou, 2015 (N = 492)	Zhengzhou, 2017 (N = 933)	Nanning, 2018 (N = 724)	Taizhou, 2019 (N = 474)
			Sex, N (%)		
male	1002 (38.2)	196 (39.8)	377 (40.4)	288 (39.8)	141 (29.7)
female	1621 (61.8)	296 (60.2)	556 (59.6)	436 (60.2)	333 (70.3)
			Age, mean (sd)		
mean (SD)	49 (13)	48 (13)	44 (13)	55 (11)	53 (9.1)
			BMI, mean (sd)		
mean (SD)	25 (3.6)	25 (3.7)	25 (3.7)	24 (3.3)	25 (3.4)
			Smoke status, mean (sd)		
never smoker	1975 (75.3)	357 (72.6)	705 (75.6)	547 (75.6)	366 (77.2)
former smoker	116 (4.4)	30 (6.1)	39 (4.2)	31 (4.3)	16 (3.4)
current smoker	532 (20.3)	105 (21.3)	189 (20.3)	146 (20.2)	92 (19.4)
			Smoke pack year mean (sd)		
mean (SD)	0.61 (1.3)	0.61 (1.3)	0.53 (1.1)	0.67 (1.4)	0.63 (1.3)
			Passive smoking		
yes	1410 (53.8)	279 (56.7)	441 (47.3)	417 (57.6)	273 (57.6)
no	1213 (46.2)	213 (43.3)	492 (52.7)	307 (42.4)	201 (42.4)
			Alcohol consumption, N (%)		
rarely	2127 (81.1)	373 (75.8)	785 (84.1)	589 (81.4)	380 (80.2)
once a week	165 (6.3)	26 (5.3)	82 (8.8)	45 (6.2)	12 (2.5)
2−3 times a week	67 (2.6)	0 (0.0)	32 (3.4)	23 (3.2)	12 (2.5)
>3 times a week	253 (9.6)	93 (18.9)	25 (2.7)	65 (9.0)	70 (14.8)
NA	11 (0.4)	0 (0.0)	9 (1.0)	2 (0.3)	0 (0.0)
			Education, N (%)		
uneducated	224 (8.5)	83 (16.9)	22 (2.4)	34 (4.7)	85 (17.9)
primary	594 (22.6)	126 (25.6)	108 (11.6)	209 (28.9)	151 (31.9)
junior secondary	989 (37.7)	178 (36.2)	297 (31.8)	332 (45.9)	182 (38.4)
senior secondary	434 (16.5)	59 (12.0)	205 (22.0)	134 (18.5)	36 (7.6)
tertiary and above	381 (14.5)	46 (9.3)	300 (32.2)	15 (2.1)	20 (4.2)
NA	1 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)
			Education household, N (%)		
uneducated	85 (3.2)	26 (5.3)	22 (2.4)	11 (1.5)	26 (5.5)
primary	567 (21.6)	129 (26.2)	144 (15.4)	160 (22.1)	134 (28.3)
junior secondary	1161 (44.3)	221 (44.9)	353 (37.8)	356 (49.2)	231 (48.7)
senior secondary	572 (21.8)	88 (17.9)	256 (27.4)	161 (22.2)	67 (14.1)
tertiary and above	232 (8.8)	28 (5.7)	158 (16.9)	30 (4.1)	16 (3.4)
NA	6 (0.2)	0 (0.0)	0 (0.0)	6 (0.8)	0 (0.0)
			Annual household income in CNY, N (%)		
<2500	28 (1.1)	7 (1.4)	3 (0.3)	17 (2.3)	1 (0.2)
2500−4999	37 (1.4)	7 (1.4)	1 (0.1)	27 (3.7)	2 (0.4)
5000−9999	113 (4.3)	17 (3.5)	23 (2.5)	67 (9.3)	6 (1.3)
10,000−19,999	189 (7.2)	21 (4.3)	51 (5.5)	97 (13.4)	20 (4.2)
20,000−34,999	351 (13.4)	60 (12.2)	122 (13.1)	117 (16.2)	52 (11.0)
>35,000	1647 (62.8)	376 (76.4)	721 (77.3)	223 (30.8)	327 (69.0)
NA	258 (9.8)	4 (0.8)	12 (1.3)	176 (24.3)	66 (13.9)
			Hypertension, N (%)		
yes	948 (36.1)	98 (19.9)	277 (29.7)	342 (47.2)	231 (48.7)
no	1675 (63.9)	394 (80.1)	656 (70.3)	382 (52.8)	243 (51.3)

2.3. Proteomic Quantification

All plasma samples were stored at −80 °C at the Fudan University Taizhou Institute prior to being collectively submitted for proteomic sequencing. The individual proteomic data were profiled by nanoLC−MS on a Q Exactive HF-X instrument in DIA mode (C18, 150 μm × 8 cm; 0.1% FA/water and 80% ACN/19.9% water/0.1% FA). DDA runs (n = 327) were searched with MSFragger and merged by SpectraST to build a single reference spectral library (1% FDR); DIA data were quantified with DIA-NN, and protein abundances were summarized by iBAQ and normalized as FOT (= iBAQ/total iBAQ × 10^5). A pooled plasma QC was injected every 20 runs.[27] Detailed parameters and additional information are provided in the Supporting Information (Text S1).

A total of 12,882 proteins were detected. During quality control, we removed proteins missing in >90% of participants, leaving 6528 proteins. Before statistical analyses, the retained protein abundances were normalized using the scale function to obtain Z-scores. Missing values were imputed with the minimum observed value for each protein.

2.4. Statistical Analysis

To evaluate the association between different duration of ozone exposure and each protein level, we conducted proteome-wide association studies (PWAS) using generalized linear models with the *limma* package[28] in both the pooled cohort and site-specific subgroups The "*fit*" function simultaneously tests the association between all proteins and variables, and the "*eBayes*" function applies

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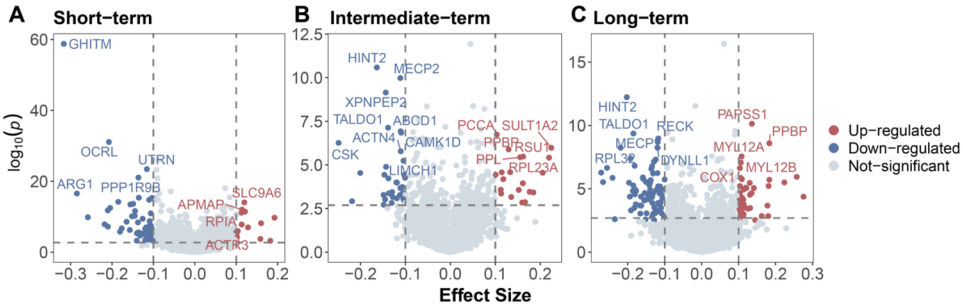

Figure 2. Results of the proteome-wide association study (PWAS) on ozone exposure. Results of the PWAS on (A) short-, (B) intermediate-, and (C) long-term ozone exposure. The line marks the threshold for statistical significance, defined as FDR < 0.05 and absolute effect size >0.1. Significant proteins are shown in two colors: red for positive effect sizes and blue for negative effect sizes.

an empirical Bayes framework that borrows information across all proteins to moderate the standard error estimates, stabilizing effect size estimation and improving the reliability of statistical results in omics data. We incorporated several covariates to adjust for confounding, which includes age, sex, smoking status (not, former, or current smoker), smoke pack year, passive smoking (yes or no), BMI, both education and household-head education (uneducated, primary, junior secondary, senior secondary, tertiary, and above), household income (<¥2500, ¥2500 - ¥4999, ¥5000 - ¥9999, ¥10,000 - ¥19,999, ¥20,000 - ¥34,999, >35,000), alcohol consumption (never, once a week, 2−3 times a week, greater than 3 times a week) sampling point index (Taizhou 2015, Zhengzhou 2017, Nanning 2018, Taizhou 2019), the first ten genetics PCs, and critically, PM2.5 exposure as a key copollutant confounder. Adjustment for the genetic PCs helps remove the influence of ancestry-related variation on baseline protein levels, thereby preventing population stratification bias and enabling more accurate identification of ozone-associated proteins.[29,30] Notably, because sampling season and region were completely collinear, we considered seasonal information to be inherently captured by the region variable. We provided a directed acyclic graph (DAG, Figure S1) to illustrate the selection of the covariates. To identify significantly associated proteins, multiple testing correction was conducted for *P* values, and we applied a false discovery rate (FDR) < 0.05 and an absolute effect size (Log Fold Change) > 0.1 as a threshold. We conducted sensitivity analyses to examine the potential confounding effects of covariate selection in the PWAS, including leave-one-out analyses for each covariate from the main model, fitting NO_2-adjusted models, additional adjustment for hypertension, and, specifically for long-term analyses, further adjustment for short-term ozone exposure. Sex-stratified analyses were also conducted to explore potential sex-specific differences in the associations between ozone exposure and plasma protein levels.

To validate the differentially expressed proteins and explore the heterogeneity, we performed the same PWAS model in each subgroup. For each protein that achieved statistical significance in the pooled analysis, we extracted its coefficient and *P* value from the subgroup model. A protein was classified as consistent when it displayed the same coefficient sign and a nominal *P* value <0.05 in at least two of the four site analyses, reflecting limited heterogeneity among subgroups.

For external validation, we compared the short-term differentially expressed proteins (DEPs) identified in our study with the proteomics results from a randomized controlled trial (RCT) of 2 h ozone exposure conducted in Shanghai.[15] And we also compared our results with the findings from an observational study assessing the associations of annual average ozone exposure with 484 plasma proteins in an elderly U.S. panel.[31] A protein was considered replicated if it showed consistent direction of association in both studies and reached statistical significance (*P* value <0.05) in our discovery PWAS.

To compare protein effects across exposure durations, we rescaled ozone exposure by its interquartile range (IQR) within each window to ensure comparability and performed PWAS using the same covariates as in the main model. We then selected proteins significantly associated with short-, intermediate-, and long-term ozone exposure (FDR < 0.05) and compared the overall magnitude using both the mean and median absolute effect sizes. For proteins overlapping across the three windows, we compared absolute effect sizes between windows using pairwise Wilcox tests.

The set of proteins significantly associated with ozone was subjected to pathway enrichment analysis to elucidate underlying biological functions. The clusterProfiler R package was utilized to test for enrichment against the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome Pathway databases, using a customized background consisting of the 6528 proteins that passed quality control and were included in the analysis. To further capture the relationships between the biological pathway and process enriched by significant proteins, we performed enrichment and network analysis by Metascape (https://metascape.org/). We selected terms with *P* values less than 0.01 and a minimum of three associated genes and then grouped them into clusters according to their similarity in gene membership. The relationships were visualized as a network plot in Cytoscape,[32] with edges connecting terms that shared a similarity greater than 0.3.[33]

Additionally, we utilized the Comparative Toxicogenomics Database (CTD; https://ctdbase.org/, data as of 30 April 2025) to conduct two types of analyses: chemical−disease interactions and gene−disease interactions. To identify proteins potentially linking ozone exposure to disease risk, we cross-referenced curated disease information for both ozone and the significant proteins detected in our PWAS.

3. RESULTS

3.1. Study Population and Exposure Levels

The final study cohort comprised 2623 individuals for whom both proteomic and ozone exposure data were available. Participants were recruited from four distinct cohorts: Taizhou in 2015 (N = 492), Zhengzhou in 2017 (N = 933), Nanning in 2018 (N = 724), and Taizhou again in 2019 (N = 474). The distributions of short-, intermediate-, and long-term ozone exposure levels for these four regions are detailed in the Supporting Information (Figure S2). The population had a mean age of 49.23 ± 12.67 years (range: 18−82), with a higher proportion of females (1,621; 61.80%) than males (1,002; 38.20%). A significant sex difference was observed in smoking habits; while the vast majority of female participants were nonsmokers (1,600; 98.70%), only 37.42% of male participants (N = 375) reported no history of smoking. A comprehensive summary of the demographic characteristics is provided in Table 1.

3.2. Proteome-Wide Association Study (PWAS)

A total of 6528 proteins were incorporated in association analysis with ozone exposure after quality control and

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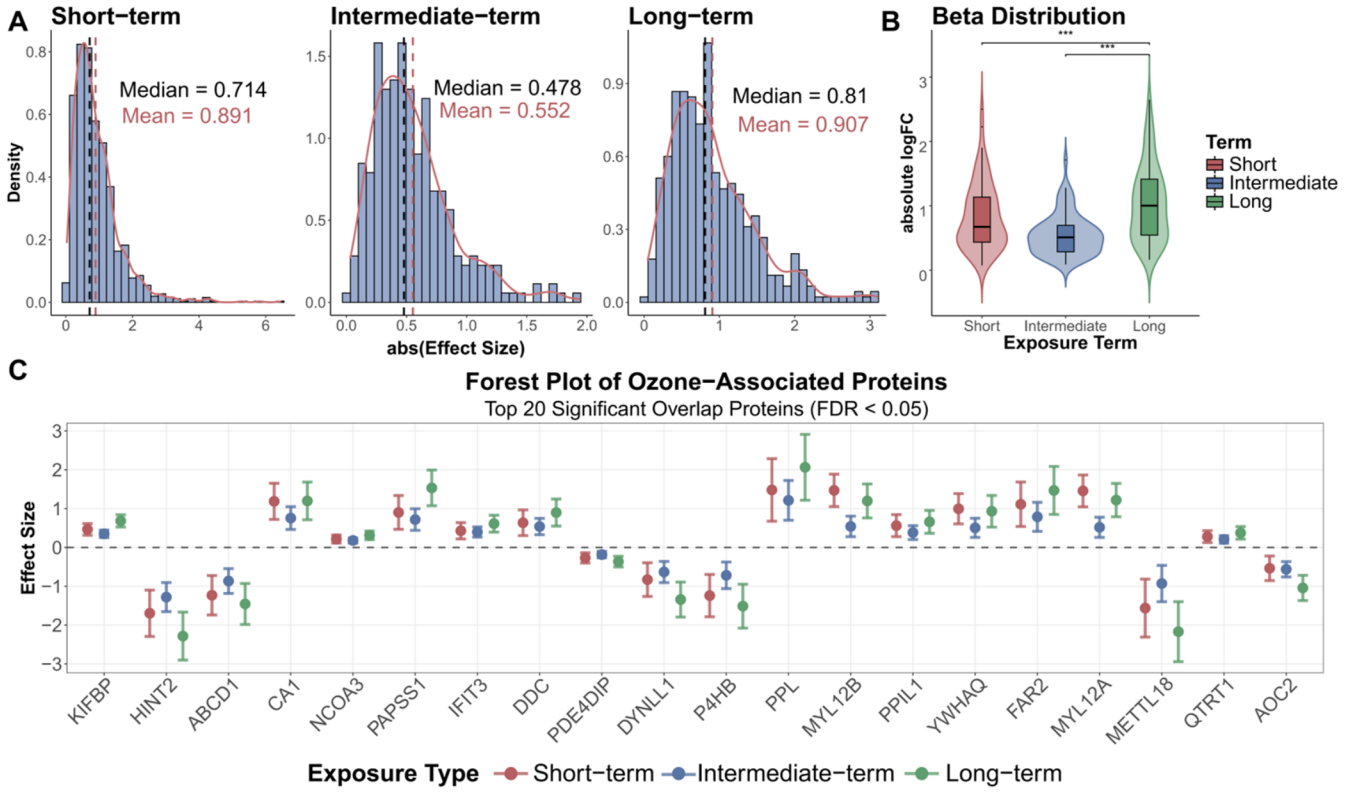

Figure 3. Comparison of effect size for proteins significantly associated with varying ozone exposure durations. (A) The density plot of the significant proteins (FDR < 0.05) associated with each duration of ozone exposure. The black line represents the median of the absolute effect size, while the red line indicates the mean. (B) The box plot shows the overlapped proteins that are significant in each ozone exposure. The Wilcox tests were performed on the absolute effect size to assess the differences (*** indicates *P* value <0.01). (C) The effect sizes of short-, intermediate-, and long-term ozone exposure at the single protein level. The top 20 proteins were selected from a larger set that were significantly associated with ozone across all time windows (FDR < 0.05 and consistent direction of effect size).

normalization. The t-SNE (t-distributed Stochastic Neighbor Embedding) clustering analysis showed distinct proteomic patterns across different spatiotemporal populations, supporting the advantages of a cohort study of capturing the common effects of ozone exposure (Figure S3). Through PWAS analysis, 209 DEPs were identified as significantly associated with ozone exposure at three ozone exposure durations (FDR < 0.05, |Effect size| > 0.1). Specifically, 74, 52, and 125 DEPs were detected under short-term (1 month), intermediate-term (1 year), and long-term (10 years) ozone exposure conditions, respectively (Figure 2 and Table S2). Four DEPs were overlapped in short- and intermediate-term analyses, five DEPs were overlapped in short- and long-term analyses, and 35 DEPs were overlapped in intermediate-term and long-term analyses. Moreover, two DEPs, *AGRN* and *ANG*, were simultaneously associated with all three ozone exposures. In addition, sex-stratified PWAS revealed more proteins significantly associated with ozone in females (n = 160) than in males (n = 88, Figure S4 and Table S3).

We performed internal and external validation to replicate the identified proteins. Overall, PWAS across four subgroups validated 42 out of the 209 DEPs, with significant effects observed in consistent directions (*P* value <0.05, Figure S5), and the validation results for each exposure duration are provided in the Supporting Information Table S4. For independent replication, we compared the 74 short-term associated proteins with the findings derived from a randomized controlled test (RCT) of ozone exposure from Niu et al. Among the 48 proteins significantly associated with 2 h ozone exposure identified through proteomic analysis, 19 proteins were replicated in our study with consistent effect directions and nominal significance level (*P* value <0.05, Supporting Information Table S5). Among these proteins, *PROZ* and *HABP2* were identified as coagulation pathway-related proteins, *APOH* was associated with lipid synthesis and metabolism, while *C1QC* and *SELL* were immune-related proteins. In the study by Tang et al., 47 proteins were significantly associated with ozone exposure (p < 0.05), 18 of which were present in our data set. Of these, two proteins—*HBQ1* and *L1CAM*—replicated in our long-term exposure analysis, and four—*L1CAM*, *PTPRM*, *LGALS9*, and *MSLN*—replicated in our short-term analysis. Functionally, *HBQ1* is a hemoglobin subunit implicated in oxygen transport and hypoxia responses; *L1CAM*, *PTPRM*, and *MSLN* participate in signal transduction and cell adhesion; *LGALS9* is linked to immune regulation. These findings corroborate our results and implicate biological pathways in the mechanisms through which ozone exerts its effects.

Sensitivity analyses confirmed that the observed associations between ozone and the identified proteins were robust. The effect sizes remained stable in the leave-one-out sensitivity analyses, where each key covariate was sequentially excluded from the main model. The effects of $PM_{2.5}$ on ozone-associated proteins were inconsistent across different exposure durations, with notable influence on short-term associated proteins but minimal impact on intermediate-term and long-term associated proteins. This result validated our approach of adjusting for $PM_{2.5}$ to isolate the specific protein responses to ozone

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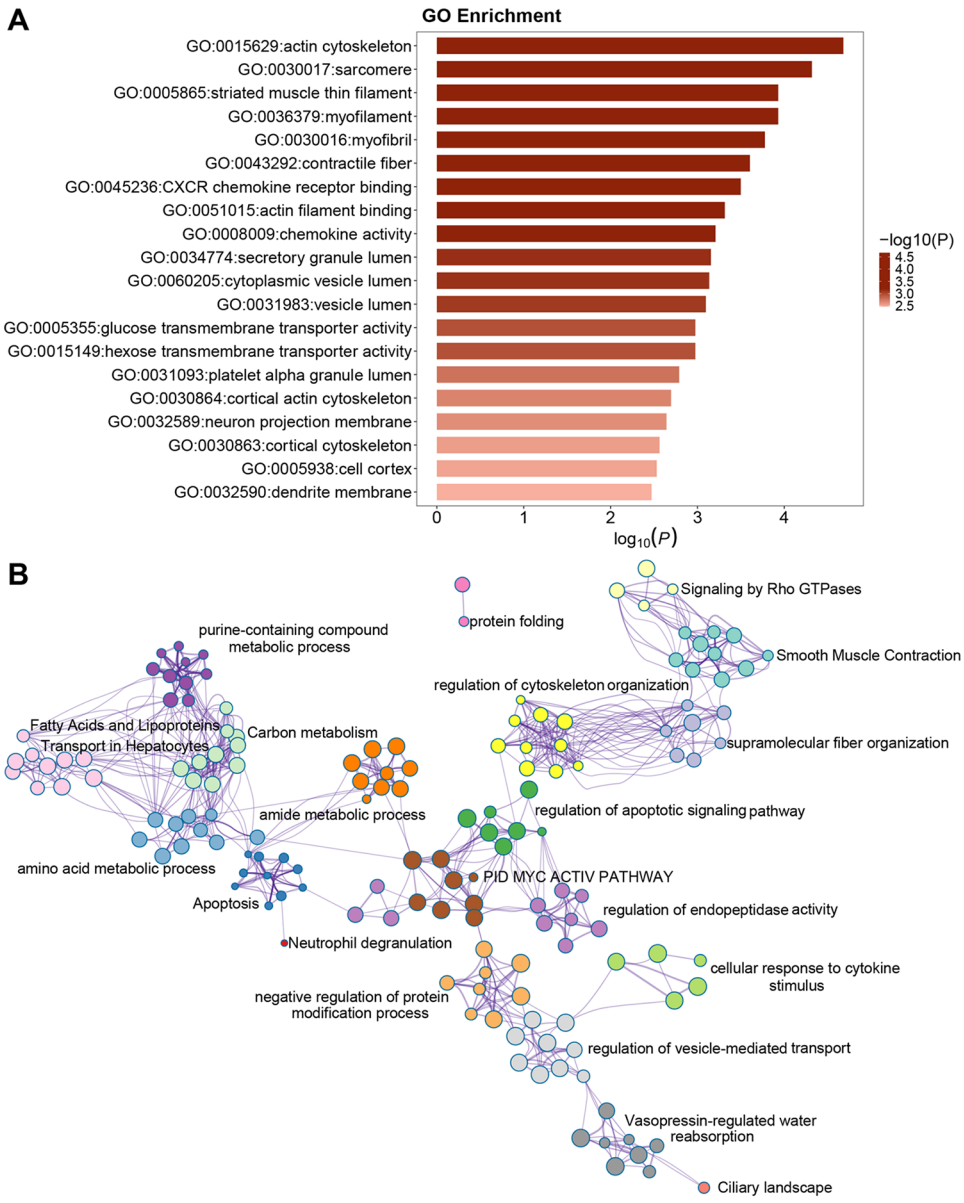

Figure 4. Enrichment of identified proteins and module clustering. (A) Results of the top 20 GO enrichment terms on the ozone-related proteins. The color indicates the $-\log_{10}(P \text{ value})$ of the biological pathway as determined by Fisher's exact test. (B) Network visualization of enriched terms generated using Metascape. Each node corresponds to an enriched term and is colored according to its cluster ID. For each cluster, a representative term is labeled to highlight the primary pathway within that group.

exposure (detailed in Supporting Information Figure S6). Moreover, additional adjustment for NO_2 yielded similar effect estimates, with overall association patterns remaining consistent except for the intermediate-term exposure (Figure S7). Further adjustments for hypertension and for short-term exposure in the long-term model also showed consistent results (Figure S8 and S9).

3.3. Proteomic Effects of Ozone Across Exposure Durations

To investigate the impact of ozone exposure duration on protein responses, we selected proteins with FDR < 0.05 in the PWAS results with ozone rescaled by the interquartile range within each exposure window (1,081 proteins in short-term, 246 in intermediate-term, and 409 in long-term) and compared their effect sizes. Results indicated that long-term ozone exposure exhibited stronger effects than short- and intermediate-term exposures (median absolute effects: 0.907 vs 0.891 and 0.552; Figure 3A).

Furthermore, among the 76 proteins that were consistently significant (consistent direction of effect size and FDR < 0.05) across all three exposure durations, pairwise Wilcox tests demonstrated significant increases in long-term duration (short-term vs long-term, P value = 0.022; intermediate-term vs long-term, P value = 3.15e-08, Figure 3B). Moreover, comparing effect sizes for each protein across all three exposure windows revealed consistently stronger long-term effects relative to short- and intermediate-term exposures (Figures 3C and S10).

3.4. Pathway Analysis of Ozone-Related Proteins

To explore the biological implications of ozone-exposure-associated proteins, we performed functional analysis for the identified proteins across three exposure durations in the

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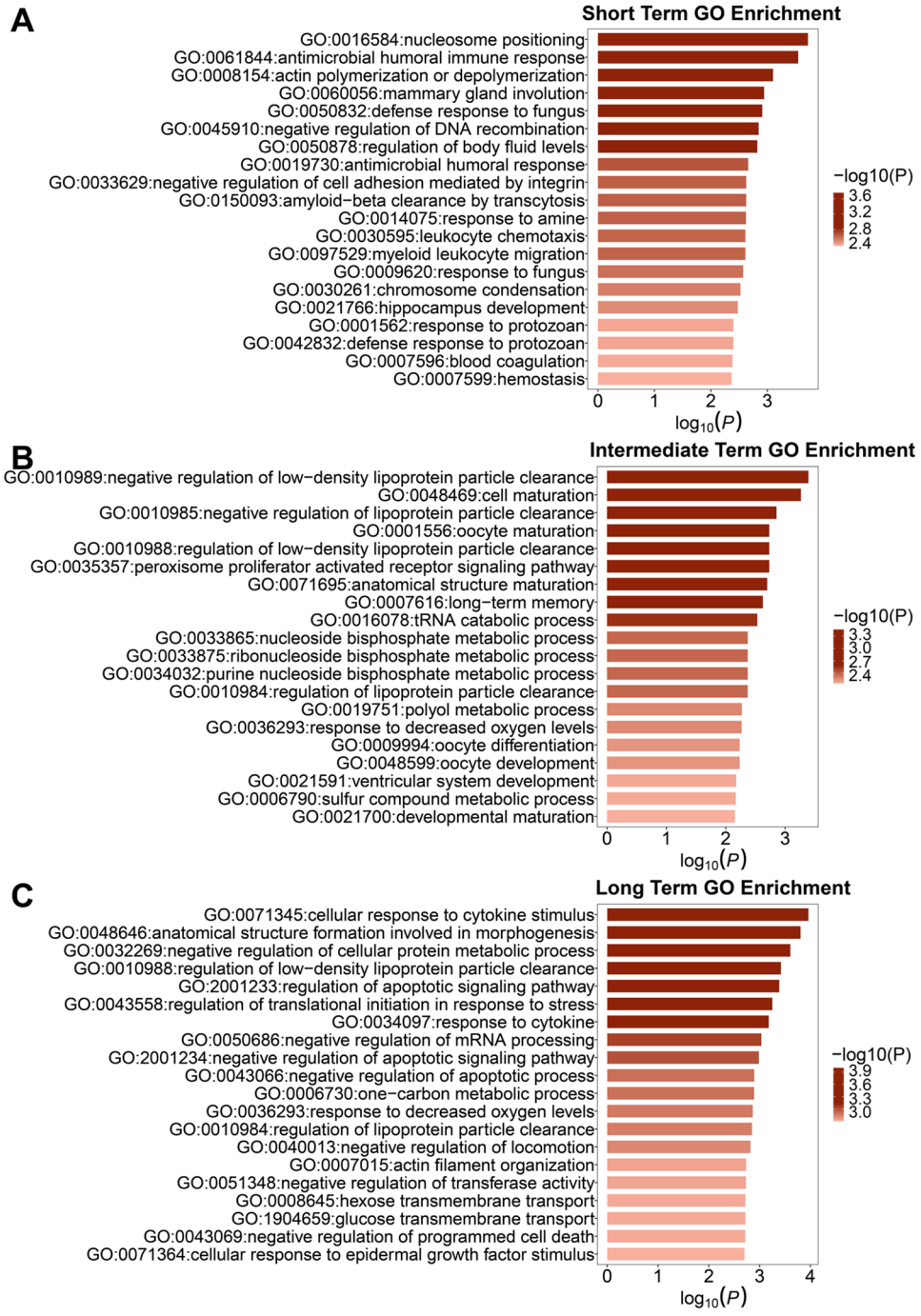

Figure 5. GO enrichment of identified proteins in three different ozone exposure duration. Top 20 enriched Biological Process (BP) terms for (A) short-, (B) intermediate-, and (C) long-term ozone exposure related-proteins (from top to bottom). The color indicates the statistical significance of the biological pathway as determined by Fisher's exact test, while the size of the dot reflects the number of proteins associated with each GO term.

primary PWAS model. A total of 209 proteins were identified and subsequently mapped to their corresponding genes. GO, KEGG, and Reactome enrichment analysis was conducted to elucidate the functional characteristics of these ozone-associated proteins. The top 20 significantly enriched GO terms, as shown in Figure 4, revealed that the ozone-associated proteins were predominantly enriched in pathways related to vesicular transport ($P_{GO:0031983}$ = 8.01e-4), actin organization ($P_{GO:0015629}$ = 2.09e-05), nucleotide metabolism ($P_{GO:1901292}$ = 1.18e-03), and platelet granules ($P_{GO:0031093}$ = 1.62e-03). Additionally, separate pathway analyses for proteins associated with each exposure duration revealed that *PF4*, *GP9*, and *LNPK* were enriched in blood coagulation ($P_{GO:0007596}$ = 0.004) under short-term ozone exposure, consistent with previous findings reported by Niu et al. *ANG* and *TUBB8* were enriched in oocyte maturation ($P_{GO:0001556}$ = 1.85e-03) specifically under intermediate-term exposure. *CA1*, *MTHFD1*, *SHMT1*, and *MAT2A* were enriched in a one-carbon metabolic process ($P_{GO:0006730}$ = 1.28e-03) specifically under long-term exposure (Figure 5). The detailed enrichment

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results for KEGG and Reactome are provided in the Supporting Information (Figure S11, Tables S10 and S11). Furthermore, Metascape-based pathway enrichment and network clustering analysis revealed four major functional clusters, including neutrophil degranulation, apoptosis, apoptosis purine metabolic process, and cytoskeleton organization (Figure 4B and Table S12). Moreover, sex-stratified PWAS enrichment analyses showed that male participants were enriched in pathways related to receptor tyrosine kinase (RTK) signaling and amyloid clearance, whereas female participants were enriched in pathways related to cellular stress−proteostasis and metabolic reprogramming (Figure S12).

Table 2 summarizes the genes significantly associated with ozone exposure levels, which were identified to be linked with known disease gene networks related to ozone exposure by using CTD tools. We identified 22 protein-coding genes associated with different types of cardiovascular, respiratory, immune, and metabolic diseases. Notably, we found that 10 proteins, *SERPINE1, PPBP, TPM1, MMP9, GSTP1, TALDO1, SLC2A4, EIF2S1, CD163, and MECP2*—corresponding to genes implicated in chronic diseases (such as hypertension, asthma, Parkinson's disease, Alzheimer's disease, insulin resistance, chronic kidney disease, rheumatoid arthritis, and COPD) appeared among the long-term ozone exposure-associated proteins. In contrast, two proteins, *IL6ST* and *PF4*, corresponding to genes related to acute inflammatory or vascular responses, were identified among the short-term ozone exposure-associated proteins. Overall, these findings suggest that the ozone-associated DEPs identified in our study are involved in a range of diseases previously reported in the literature; the detailed literature information is provided in Supporting Information Table S13.

Table 2. Validation of Differentially Expressed Proteins with Ozone-Associated Diseases Using CTD

S.no	curated diseases	no. of genes	significant proteins in PWAS
1	myocardial ischemia	*IL6ST, FABP5, SPARC, P4HB, NCL, EIF2AK3, GSTP1*	7
2	hypertension	*SERPINE1, PPBP, TPM1, MMP9, GSTP1*	5
3	asthma	*ARG1, MMP9, GSTP1*	3
4	parkinson disease	*TALDO1, RPL23A, GSTP1*	3
5	weight gain	*IGFBP5, SPARC, SPTBN1*	3
6	alzheimer disease	*SLC2A4, EIF2S1*	2
7	insulin resistance	*CD163, SLC2A4*	2
8	kidney diseases	*SPARC, GSTP1*	2
9	lung neoplasms	*PPBP, GSTP1*	2
10	myocardial infarction	*LDHA, MMP9*	2
11	pneumonia	*IL6ST, PF4*	2
12	pulmonary fibrosis	*MECP2, MMP9*	2
13	acute kidney injury	*GSTP1*	1
14	arthritis, rheumatoid	*IL6ST*	1
15	atherosclerosis	*SERPINE1*	1
16	heart failure	*SERPINE1*	1
17	hyperglycemia	*CD163*	1
18	inflammation	*MMP9*	1
19	pulmonary disease, chronic obstructive	*MMP9*	1
20	respiratory hypersensitivity	*SERPINE1*	1

4. DISCUSSION

We believe this is the first study to systematically characterize proteomic changes linked to ozone exposure over various durations in a multicenter cohort of Chinese participants. The NSPT cohort included individuals recruited from suburban areas in Zhengzhou, Nanning, and Taizhou, representing northern, southern, and eastern China, respectively, thereby capturing both regional characteristics and population heterogeneity within the Chinese population. By employing an untargeted proteomic approach, we analyzed the associations between short-term (1 month), intermediate-term (1 year), and long-term (10 years) ozone exposure and 6528 plasma proteins. PWAS identified 209 proteins that were significantly linked to ozone exposure, with the effect on protein strengthening as the exposure duration increased. Enrichment results showed that these proteins were associated with coagulation, vesicular transport, actin organization, and nucleotide and energy metabolism. Specifically, short-term exposure was enriched in the coagulation pathway, while intermediate-term exposure was enriched in the peroxisome pathway.

PWAS results revealed proteins significantly associated with ozone exposure. Sensitivity analyses revealed that copollutants partially influenced the effect sizes of ozone-related DEPs, likely due to the correlation between ozone and other ambient pollutants, underscoring the importance of accounting for copollutant adjustment in ozone−protein association analyses. Associations remained consistent after controlling for demographic, lifestyle, and socioeconomic covariates. Furthermore, by validating the proteins identified in our short-term exposure analysis with those reported in the ozone proteomics RCT study by Niu et al., we found 19 proteins significantly associated in both studies. Despite differences in experimental design, this overlap revealed the robustness of our statistical approach in population-based cohort studies and highlighted the consistency of our findings.

Our results indicated that ozone-related proteins are associated with platelet granules, which indicates a link to coagulation function. Multiple studies have demonstrated that ozone exposure affects coagulation function.[34−36] A previous meta-analysis found that short-term exposure to ambient ozone was significantly associated with elevated levels of PAI-1 as well as P-selectin.[37] A recent study investigated the mediating effect of DNA methylation in the relationship between air pollution, including ozone, and coagulation biomarkers.[38] Niu et al. found that a 2 h short-term ozone exposure significantly increased the expression of procoagulant proteins while reducing the expression of anticoagulant proteins, suggesting that short-term ozone exposure triggers a hypercoagulant function.[15] Coincidentally, the proteins significantly associated with short-term ozone exposure in our study were also notably enriched in coagulation pathways. Among these findings, the anticoagulant protein *PF4* (Platelet Factor 4) was observed to be negatively associated with short-term ozone exposure. Furthermore, the observed associations of the anticoagulant protein *PROZ* (Protein Z) and the procoagulant protein *HABP2* (Hyaluronan-Binding Protein 2) were consistent in direction with those reported by Niu et al. Notably, we also identified a link between long-term ozone exposure and coagulation function, characterized by a

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significant enrichment of proteins within the platelet granule lumen. This group included key procoagulant proteins such as *SERPINE1* (Plasminogen Activator Inhibitor-1) and PPBP (Platelet Basic Protein). Collectively, these results further support the role of ozone exposure, particularly in the short term, in promoting hypercoagulability, as demonstrated by our PWAS findings and pathway enrichment analyses in this population-based study.

Results from the enrichment analysis of the significant proteins also revealed that vesicle trafficking pathways play an important role at different exposure durations. In mice, repeated ozone inhalation altered vesicle and nonvesicle airway proteins linked to mucus-inflammatory lung disease via vesicle-pathway disruption.[39] In addition, ozone or particulate exposure likewise compromises epithelial-barrier integrity by disturbing vesicle trafficking and intercellular junctions.[40] Although direct epidemiological evidence linking vesicle trafficking to air pollution remains limited, existing studies indicate that extracellular vesicle (EVs) may mediate the adverse health effects associated with air pollution exposure,[41,42] and this study provides the first human support. Overall, our study demonstrates that ozone exposure may induce alterations in vesicle trafficking, which could play a critical role in the effects of air pollution on chronic respiratory, cardiovascular, and neurologic diseases.[43,44]

Moreover, our results also suggest that ground-level ozone exposure leads to alterations in nucleotide metabolism pathways in the human body. This is consistent with previous findings, as ozone exposure has been shown to cause oxidative damage to DNA, leading to the formation of oxidized nucleotides, such as 8-oxoguanine. Such oxidative stress can result in mutations and compromise genomic integrity.[45] Nucleotides, as fundamental building blocks of genetic material, play a crucial role in the mechanisms of DNA damage and repair processes. Previous studies utilizing metabolomic approaches have identified that short-term ozone exposure is associated with perturbations in pyrimidine metabolism.[46] In addition, one study has shown that ambient ozone is associated with increased concentrations of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of DNA oxidative damage.[47] Among the 18 genes enriched in GO:0009117 (nucleotide metabolic process), *NT5C* encodes a cytosolic 5′,3′-nucleotidase that catalyzes the dephosphorylation of deoxyribonucleotides, while *NME1* encodes a nucleoside diphosphate kinase responsible for catalyzing the transfer of phosphate groups between nucleoside diphosphates and ATP. Alterations in the abundance of these catalytic enzymes offer direct molecular evidence of ozone-induced genomic stress and further underscore the unique ability of proteomic analyses to reveal mechanistic insights into cellular responses.

The disease effects of ozone have been well established in numerous epidemiological studies; however, the key factors driving these effects remain unclear. We utilized the CTD database to examine evidence linking DEPs to ozone-related disease outcomes and noted that DEPs associated with cardiovascular diseases, respiratory diseases, and immune disorders have previously been reported (Table 2). For example, *MMP9* has been implicated in chronic obstructive pulmonary disease (COPD) and inflammation and is related to phosphorylation pathways.[48−50] *GSTP1*, on the other hand, has been shown to play a role in hypertension, myocardial ischemia, and asthma,[50−52] and *IL6ST* has been associated with the incidence of rheumatoid arthritis and pneumonia.[53,54] Our study offers valuable insights into the pathogenic mechanisms underlying ozone exposure.

Besides highlighting specific proteins and pathways, our research revealed novel insights into the effects of ozone exposure on plasma proteomic profiles over 1 month, 1 year, and 10 year periods. Previous studies have rarely examined multiple ozone exposure durations simultaneously. Our study identified unique sets of proteins linked to short-, intermediate-, and long-term ozone exposure, noting that the intermediate- and long-term groups had the highest degree of overlap. This pattern is consistent with our earlier findings in DNA methylation studies.[55] Existing research indicates that short-term ozone exposure (2 or 24 h) induces inflammation, acute oxidative stress, and coagulation changes,[11,15] while long-term exposure is linked to chronic declines in lung and neurological function.[56−58] Together with our PWAS results, these findings suggest that short- and long-term ozone exposures have distinct underlying mechanisms. Moreover, comparison of significant proteins' effect sizes showed that long-term ozone exposure exerted the strongest effects, highlighting a lasting effect and potential shared pathways. For instance, *ANG*, a protein consistently identified across all exposure durations, is involved in angiogenesis and cellular stress responses and has been repeatedly associated with cardiovascular disease risk.[59−61] Additionally, under an FDR < 0.05 threshold, we detected a larger number of proteins associated with short-term ozone exposure; however, most effect sizes were modest. This pattern suggests that acute, short-term exposure elicits transient yet widespread stress responses, particularly inflammatory and vascular changes,[62] whereas with prolonged exposure, adaptive regulatory processes may emerge, yielding more persistent disruptions of cellular homeostasis that are more closely linked to disease risk.[63]

The present study has several strengths. First, we employed a nontargeted proteomics approach to comprehensively and systematically investigate the associations between ozone exposure and protein expression. This method allowed us to move beyond a limited set of known proteins, expanding the scope of the protein markers included in the analysis. By incorporation of omics-based statistical analyses, this approach improved the statistical power and reliability of the signals compared to traditional analyses. Second, by utilizing a validated environmental ozone prediction model, we obtained large-scale cohort data on ozone air pollution exposure for short-, intermediate-, and long-term durations, all at relatively low cost. This design made it possible to direct comparisons of the effects of ozone exposure across different time windows. Finally, our study population was drawn from three regions across four spatiotemporal settings in China. Despite the genetic and lifestyle patterns differing across regions, this diversity provided robust support for identifying the common effects of ozone exposure on protein expression.

However, several limitations need to be acknowledged. First, individual ozone exposure was predicted from residential information using a machine-learning model, which likely estimates ambient ozone levels at a given location over a specified time window. If participants changed residence during the 10 year window, this approach may introduce exposure misclassification, particularly for long-term assessments. Moreover, ground-level ozone exposure can be modulated by the frequency of outdoor activities, potentially

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adding measurement error. Future studies should collect data on residential mobility and outdoor activity patterns. In addition, for ozone prediction models, future work should employ more refined exposure-assessment methods—such as satellite-derived pollutant products or high-resolution spatiotemporal models—to better capture within-city gradients and personal exposure. Second, the absence of transcriptomic and metabolomic data in the current NSPT cohort constrains the analysis to protein enrichment approaches for elucidating associations with biological mechanisms. This limitation precludes the investigation of the regulatory effects of significantly associated proteins on gene expression and metabolite profiles, thereby restricting a more comprehensive understanding of the biological processes influenced by ozone exposure. Third, as the NSPT is an observational natural population cohort study without disease outcome data and given the current lack of cross-cohort validation and consistent exposure time windows, there are limitations to the current evidence. More studies are needed to replicate the proteomic effects of ozone exposure and clarify its disease effects. Finally, although the associations appear predominantly linear within the ozone concentration range observed in this population,[26] we do not exclude the possibility of nonlinear relationships at higher ozone levels, which will be an important direction for future research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.5c10322.

(1) Plasma protein profiling (Text S1); (2) proteome-wide association study (PWAS) (Text S2); (3) sex-stratified proteome-wide association analyses of ozone exposure (Text S3); (4) stratified analyses by sampling site to examine the heterogeneity of PWAS (Text S4); (5) sensitivity analyses to assess the robustness of the PWAS signals (Text S5); (6) KEGG and reactome enrichment analysis (Text S6); (7) a directed acyclic graph (DAG) illustrating the relationships among ozone exposure, plasma protein levels, and covariates (Figure S1); (8) ozone exposure in NSPT cohort and subgroups and relationships between ozone exposure and covariables (Figure S2), (9) score plot of t-SNE analysis of proteomic data for all individuals (Figure S3); (10) sex-stratified results of the proteome-wide association study (PWAS) on ozone exposure (Figure S4), (11) effect size of validated proteins across subgroups revealed heterogeneity (Figure S5); (12) effect size estimates of associated proteins from primary model, leave-one-covariate-out models for sensitivity analysis (Figure S6); (13) sensitivity analysis with additional adjustment for NO_2 (Figure S7); (14) sensitivity analysis with additional adjustment for hypertension (Figure S8); (15) sensitivity analysis of the long-term ozone exposure PWAS, additionally adjusted for short-term ozone exposure (Figure S9); (16) comparison of effect sizes for proteins significantly shared across short-, intermediate-, and long-term ozone exposure (Figure S10), (17) KEGG and reactome enrichment results of all 209 ozone-related proteins (Figure S11), (18) sex-specific enrichment analysis of ozone-associated proteins (Figure S12) (PDF)

(1) questionnaire for collecting residential information (Table S1); (2) summary table of PWAS (Table S2); (3) summary table of PWAS after sex-stratification (Table S3); (4) summary table of PWAS after group-stratification (Table S4); (5) replication of identified proteins in the previous ozone RCT study of Yue Niu et al. 2022 (Table S5); (6) enriched pathways of all identified ozone-related proteins (Table S6); (7) enriched pathways of short-term ozone-related proteins (Table S7); (8) enriched pathways of intermediate-term ozone-related proteins (Table S8); (9) enriched pathways of long-term ozone-related proteins (Table S9); (10) KEGG pathways of all identified ozone-related proteins (Table S10); (11) reactome terms of all identified ozone-related proteins (Table S11); (12) pathway network and clusters by metascape (Table S12); (13) curated diseases in relation to ozone exposure using CTD tools (Table S13) (XLSX)

■ AUTHOR INFORMATION

Corresponding Authors

Haidong Kan − *School of Public Health, Shanghai Institute of Infectious Disease and Biosecurity, Key Lab of Public Health Safety of the Ministry of Education and NHC Key Lab of Health Technology Assessment, Fudan University, Shanghai 200433, China; Children's Hospital of Fudan University, National Center for Children's Health, Shanghai 200433, China;* orcid.org/0000-0002-1871-8999; Email: kanh@fudan.edu.cn

Sijia Wang − *Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China*; Email: wangsijia@sinh.ac.cn

Authors

Kaixuan Li − *Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China;* orcid.org/0000-0003-2869-0050

Wenran Li − *Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China;* orcid.org/0000-0002-1712-6895

Yue Niu − *School of Public Health, Shanghai Institute of Infectious Disease and Biosecurity, Key Lab of Public Health Safety of the Ministry of Education and NHC Key Lab of Health Technology Assessment, Fudan University, Shanghai 200433, China*

Xiyang Cai − *Guangzhou National Laboratory, No. 9 XingDaoHuanBei Road, Guangzhou International Bio Island, Guangzhou 510005 Guangdong,, China*

Xia Meng − *School of Public Health, Shanghai Institute of Infectious Disease and Biosecurity, Key Lab of Public Health Safety of the Ministry of Education and NHC Key Lab of Health Technology Assessment, Fudan University, Shanghai 200433, China;* orcid.org/0000-0002-0751-1722

Li Jin − *State Key Laboratory of Genetic Engineering, Collaborative Innovation Center for Genetics and Development, and Human Phenome Institute and Ministry of Education Key Laboratory of Contemporary Anthropology, Department of Anthropology and Human Genetics, School of Life Sciences, Fudan University, Shanghai 200433, China*

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Complete contact information is available at:
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Author Contributions

[▽]K.L., W.L., and Y.N. contributed equally to this work. S.W., H.K., and K.L. conceived and designed the study. K.L. and W.L. did statistical analyses and wrote the manuscript. Y.N., X.C., X.M., and L.J. contributed to data collection and the overall structure of the study. S.W. and H.K. reviewed and edited the manuscript. S.W. and H.K. supervised the study. All authors have read and approved the final draft of the paper.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ABCD1, ATP-binding cassette subfamily D member 1
AGRN, agrin
ANG, angiogenin
APOH, apolipoprotein H
ARG1, arginase-1
C1QC, complement C1q subcomponent subunit C
CTD, comparative toxicogenomics database
CD163, cluster of differentiation 163
DEPs, differentially expressed proteins
EIF2AK3, eukaryotic translation initiation factor 2 α kinase 3
EIF2S1, eukaryotic translation initiation factor 2 subunit 1
EVs, extracellular vesicles
FABP5, fatty acid binding protein 5
FAR2, fatty acyl-CoA reductase 2
GP9, glycoprotein IX
GSTP1, glutathione S-transferase Pi 1
HABP2, hyaluronan-binding protein 2
HBQ1, hemoglobin subunit theta-1
IGFBP5, insulin-like growth factor binding protein 5
IL6ST, interleukin 6 Signal Transducer
LDHA, lactate dehydrogenase A
LGALS9, Galectin-9 L1CAM L1 cell adhesion molecule
LNPK, lunapark
MECP2, methyl-CpG binding protein 2
MMP9, matrix metalloproteinase 9
MSLN, mesothelin
NCL, nucleolin
NME1, nucleoside diphosphate kinase 1
NT5C, cytosolic 5′,3′-nucleotidase
P4HB, prolyl 4-hydroxylase subunit β
PAI-1, plasminogen activator inhibitor-1 P-selectin platelet selectin
PF4, platelet factor 4
PPBP, pro-platelet basic protein
PROZ, protein Z
PTPRM, receptor-type tyrosine-protein phosphatase Mu
PWAS, proteome-wide association study
RPL23A, ribosomal protein L23a
SELL, L-selectin
SERPINE1, plasminogen activator inhibitor-1 PAI-1
SLC2A4, solute carrier family 2 member 4
SPARC, secreted protein acidic and cysteine rich
SPTBN1, spectrin β nonerythrocytic 1
TALDO1, transaldolase 1
TPM1, tropomyosin 1

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