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RESEARCH ARTICLE

Proteomic analysis of serum in workers exposed to diesel engine exhaust

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Abstract

Diesel engine exhaust (DEE) is classified as a Group 1 human carcinogen. Using a targeted proteomics approach, we aimed to identify proteins associated with DEE and characterize these markers to understand the mechanisms of DEE-induced carcinogenicity. In this cross-sectional molecular epidemiology study, we measured elemental carbon (EC) using a personal air monitor and quantified 1317 targeted proteins in the serum using the SOMAScan assay (SOMALogic) among 19 diesel exposed factory workers and 19 unexposed controls. We used linear regressions to identify proteins associated with DEE and examined their exposure-response relationship across levels of EC using linear trend tests. We further examined pathway enrichment of DEE-related proteins using MetaCore. Occupational exposure to DEE was associated with altered levels of 22 serum proteins (permutation $p < .01$). Of these, 13 proteins (CXCL11, HAPLN1, FLT4, CD40LG, PES1, IGHE.IGK..IGL, TNFSF9, PGD, NAGK, CCL25, CCL4L1, PDXK, and PLA2G1B) showed an exposure-response relationship with EC (p trend < .01), with serum levels of all but PLA2G1B declining with increasing air levels of EC. For instance, C-X-C Motif Chemokine Ligand 11 (CXCL11) showed the most significant association with DEE ($\beta = -0.25$; permutation

Mohammad L. Rahman, Bryan A. Bassig, and Yufei Dai contributed equally to this study.

Debra T. Silverman, Roel Vermeulen, Nathaniel Rothman, Yuxin Zheng, and Qing Lan cosupervised this study.

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[Correction added on January 08, 2022, after first online publication: Corresponding author Mohammad L. Rahman's e-mail ID is changed.]

$p = .00004$), where mean serum levels were 4121.1, 2356.7, and 2298.8 relative fluorescent units among the unexposed, lower exposed (median, range : 56.9, 40.2–62.1 μg/m^3 EC), and higher exposed (median, range of EC: 72.9, 66.9–107.7 μg/m^3 EC) groups, respectively (p trend $= .0005$). Pathway analysis suggested that these proteins are enriched in pathways related to inflammation and immune regulation. Our study suggests that DEE exposure is associated with altered serum proteins, which play a role in inflammation and immune regulation.

KEYWORDS
carcinogenesis, diesel engine exhaust, elemental carbon, lung cancer, proteomics, SOMAscan

1 | INTRODUCTION

Diesel engine exhaust (DEE) is a mixture of gaseous and particulate matter produced during the combustion of diesel fuel. DEE particulate matter consists of a solid elemental carbon (EC) core adhered to organic carbon compounds such as polycyclic aromatic hydrocarbons (PAHs) on the surface. DEE and its components are classified by the International Agency for Research on Cancer as a Group 1 human carcinogen based on sufficient evidence of carcinogenicity to the human lung (International Agency for Research on Cancer, 2014). Exposure to DEE has also been associated with bladder cancer (International Agency for Research on Cancer, 2014; Koutros et al., 2020; Vermeulen et al., 2014) and several noncancerous cardiovascular and pulmonary health conditions (Costello et al., 2018; Ferguson et al., 2020; Pedeli et al., 2011; Peters et al., 2004; Riedl & Diaz-Sanchez, 2005; Silverman et al., 2012). However, the underlying molecular mechanisms by which DEE exposure may lead to adverse health effects are not well-understood.

Among many constitutes of DEE, PAHs have been widely studied in relation to lung carcinogenicity. These studies suggest that the metabolism and detoxification of PAHs can produce genotoxic intermediates that can react with the DNA to form DNA adducts and in turn, play a major role in DEE-induced carcinogenicity (International Agency for Research on Cancer, 2014). DEE may also increase the production of reactive oxygen species (ROS) resulting from particle-elicited inflammation, which in turn may lead to oxidative stress-related damage to the DNA (Jantzen et al., 2012). Growing evidence suggested a link between chronic inflammation and cancers (Balkwill & Mantovani, 2001; Hanahan & Weinberg, 2011). Specifically, several recent studies have identified circulatory immune and inflammatory markers associated with lung cancer (Gomes et al., 2014; Lim et al., 2020; Pine et al., 2011; Shiels et al., 2013).

We previously conducted a cross-sectional molecular epidemiological study of diesel engine factory workers in China and examined molecular changes associated with occupational exposure to DEE (Bassig et al., 2017; Dai et al., 2018; Drizik et al., 2020; Lan et al., 2015). We reported that the number of CD4+, CD8+, and B lymphocyte subsets in the peripheral blood were increased in DEE exposed workers compared to unexposed controls (Lan et al., 2015). Recently, we conducted a genome-wide transcriptome profiling of nasal epithelium between DEE exposed workers and controls and identified 225 differentially expressed genes, primarily involved in pathways related to oxidative and endoplasmic stress, hypoxia, DNA damage, and circadian rhythm (Drizik et al., 2020). Additional analyses from this study using a targeted approach showed significant alterations in specific immune/inflammatory markers and serum cytokine levels that were linked to increased lung cancer risk (Bassig et al., 2017; Dai et al., 2018; Shiels et al., 2013, 2017). While a few studies have examined the effects of DEE on global protein changes (proteome); existing studies are limited to cell culture in vitro studies (Chiang et al., 2013; Hooven & Baird, 2008; Xiao et al., 2003) or animal models (Lewis et al., 2007). To our knowledge, no population-based study has examined global proteomic changes associated with DEE exposure.

Here, we applied a targeted proteomics assay, SOMAscan (SOMALogic) to identify serum proteins associated with DEE exposure in a well-characterized worker population (Lan et al., 2015). SOMAscan® is an aptamer-based highly sensitive and reproducible proteomics platform that can quantify over 1300 targeted proteins involved in a wide range of biological processes, including inflammation and carcinogenesis (Kim et al., 2018).

2 | MATERIALS AND METHODS

2.1 | Study population

Details of the cross-sectional molecular epidemiology study have been described elsewhere (Lan et al., 2015). In brief, we recruited 54 healthy male subjects from a diesel engine testing facility in China; all of whom spent most of their shift in direct proximity to the engine being tested. We also recruited 55 healthy male control subjects from facilities in the same geographic region with no apparent source of DEE and general dust exposure. Selected facilities included a bottling department of a brewery, a water treatment plant, a meat packing facility, and an administrative facility. A detailed walk-through survey was conducted in each of the control workplaces and no apparent sources of DEE exposure were identified. Subjects unexposed to DEE

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(i.e., controls) were frequency-matched to exposed workers on age (±5 years) and smoking status (current, former, never). Demographic and lifestyle characteristics were obtained for each worker through a structured questionnaire as part of a regular health examination conducted by the local Center for Disease Control in China. Participation in the study was voluntary for all subjects enrolled in the study, and written informed consent was obtained.

This study was approved by the Institutional Review Boards at the U.S. National Cancer Institute (NCI) and the National Institute of Occupational Health and Poison Control in the Chinese Center for Disease Control and Prevention (Protocol number 13CN101).

2.2 | Exposure assessment

Details of exposure assessment were described elsewhere (Lan et al., 2015). Briefly, air monitoring was used to assess exposure to DEE among workers in the diesel engine testing facilities. Assessment of repeated full-shift (8 h/day) personal air exposure was measured using personal cyclone air sampling equipment attached to the lapels of diesel factory workers near the breathing zone with an aerodynamic cut-off of 2.5 mm (PM2.5) at a flow rate of 3.5 L/min using quartz filters. We measured EC, a major component of DEE particulate matter, as a proxy for DEE exposure in occupational settings using NIOSH Method 5040 (National Institute for Occupational Safety and Health, 2003).

Exposure to EC in controls was measured in a subset of DEE unexposed workers ($n = 7$) per factory, except for the beer factory where no measurements could be obtained. EC exposure levels were averaged (geometric mean) by factory and assigned to all controls in that factory. Because minimal variation between factories was observed, control workers of the beer factory were assigned the average exposure of all factories. Finally, EC exposure in controls (who had no known occupational exposure to DEE) was used as a proxy for background outdoor exposure in the region to adjust for EC levels among all study participants.

2.3 | Sample collection and preparation

Peripheral blood samples were collected from all workers immediately following their work shift. Blood samples were centrifuged for 10 min at up to 1300*g* to separate the serum and stored in a -80°C freezer until further analysis. The same sample collection procedures and processing lab were used for all subjects in the study, including both the exposed and unexposed groups.

2.4 | Serum proteomic analysis

For the proteomics assay, we selected 20 DEE exposed and 20 matched unexposed workers (of a total of 54 exposed and 55 unexposed workers). Exposed workers were selected from the upper tail of the distribution of EC to evaluate associations at higher levels of exposure. A total of 1317 targeted proteins (47% secreted proteins, 28% extracellular domains, and 25% intracellular proteins) were measured using the SOMAscan 1.3k assay (SOMALogic) that required 50 μL of serum (Rohloff et al., 2014). These proteins belong to wide biological subgroups including receptors, kinases, cytokines, proteases, growth factors, protease inhibitors, hormones, and structural proteins. Each target protein had a corresponding aptamer reagent, called SOMAmer (short, single-stranded deoxyoligonucleotide) that was hybridized with high specificity. After washing and staining, the slides were scanned with an Agilent SureScan microarray scanner at a 5-m resolution to detect Cy3 fluorescence. Gridding and analysis of images were performed using Agilent Feature Extraction version 10.7.3.1 using the SOMAscan protocol.

The SOMAscan raw data were processed following the manufacturer's guidelines (Candia et al., 2017). In brief, hybridization normalization was performed using control probes to remove intra-array hybridization variation. Median signal normalization was performed within wells and SOMAmers of the same dilution group to remove sample-to-sample differences within a group. Samples were analyzed in four plates in three independent runs. Three quality control pooled samples were run in triplicate on each plate. Five replicates of calibrator control pooled samples were included in each plate along with study samples; the median values of calibrators were used to perform intensity scaling between plates. The average coefficient of variation for at least 50% of the proteins was below 5.4% and for 95% of the proteins was below 15.1%.

2.5 | Statistical analysis

Of 40 study participants, two (one exposed and one unexposed participant) were excluded as suspected outliers based on sample clustering and principal component analysis (Figure S1). The final analysis included 1317 targeted proteins from 19 exposed workers and 19 unexposed controls. Data were logarithm to base 10 transformed to achieve normality. All computations were performed in R statistical programming environment (version 4.0.2).

The intensity of each protein (relative fluorescence unit [RFU] in continuous scale) was compared between DEE exposed and unexposed workers using multivariable linear regression models, where each protein (continuous) was included in the model one at a time, adjusted for age (continuous), current smoking status (yes, no), and body mass index (BMI; continuous). The R package "lmPerm" was used to fit and test linear models with permutation tests using the exact method (Good, 1994). The permutation *p* value represents the probability of obtaining a result at least as extreme as the test statistics given that the null hypothesis is true. Significant associations were defined by a permutation $p < .01$. To account for multiple testing, we used Benjamini Hochberg's false discovery rate (FDR) (Benjamini et al., 2001). We also considered recent infection and current alcohol intake as potential confounders. However, our final models did not include recent infection and current alcohol use as covariates, as these

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variables were not associated with DEE exposure to confound our associations (Hernán et al., 2002). Indeed, additional adjustments for recent infection and alcohol intake did not materially change our associations.

For proteins that showed significant associations with DEE, we further examined their exposure-response relationship with EC using a linear trend test across categories of EC (0 = unexposed controls; 1 = exposed below the median concentration, 66.9 μg/m^3; range: 40.2–62.1 μg/m^3; 2 = exposed above the median, range: 66.9–107.7 μg/m^3), adjusted for covariates. We plotted mean serum levels of DEE-related proteins across categories of EC using box and whisker plots. We tested for mean differences across categories of EC using the Student's *t*-test.

To understand the system-level molecular response to DEE exposure, we calculated Spearman's rank correlation coefficients for all possible pairs of DEE-related serum proteins and constructed correlation-based networks with an effect size of Spearman's $|\rho| \geq 0.25$ separately for the exposed and unexposed groups using Cytoscape (Shannon et al., 2003). Due to the use of effect size for prioritizing network connectivity, correction for multiple hypothesis testing was not applied. Finally, to understand the underlying molecular mechanisms of DEE carcinogenicity, we conducted pathway enrichment analysis with DEE-related proteins using *MetaCore*, v20.3 (Thomson Reuters; https://portal.genego.com/). Our primary analysis included 22 DEE-related proteins that passed the significance threshold of permutation $p < .01$. As a secondary analysis, we relaxed our selection criteria (permutation $p < .05$) to include 126 DEE-related proteins in the pathway analysis. Enriched pathways were considered noteworthy if *p* values corrected for multiple testing using FDR were <0.05 (Benjamini et al., 2001).

3 | RESULTS

3.1 | Study population

Demographic characteristics of exposed and unexposed participants were shown in Table 1 and were comparable with regard to age, BMI, current smoking status, and recent infection (Table 1). The median (range) number of years exposed participants worked at the diesel testing facilities was 17.8 (1.1–28.5) years and unexposed participants at control factories was 16.7 (1.8–31.5) years. There was a wide range of exposure to EC among the exposed workers (median, range: 66.9, 40.2–107.7 μg/m^3).

3.2 | Serum proteins associated with DEE exposure

Occupational exposure to DEE was significantly associated with altered levels of 22 serum proteins (CXCL11, HAPLN1, FLT4, CD40LG, PES1, PGD, P4HB, SHH, CA3, TNFSF9, CCL20, SPINT1, PPID, ANGPT2, IGHE.IGK..IGL., CCL25, PDXK, PPY, PLA2G1B, NAGK, CCL5, CCL4L1; permutation $p < .01$) (Table 2). Of these, serum levels of all but three proteins (SPINT1, PLA2G1B, CA3) were significantly lower among DEE exposed workers compared to controls. The smallest *p* value was observed for C-X-C Motif Chemokine Ligand 11 (CXCL11; $\beta = -0.25$, permutation $p = .00004$, FDR $= 0.04$), whereas the largest effect size was observed for immunoglobulin heavy constant epsilon/immunoglobulin kappa locus/immunoglobulin lambda locus (IGHE.IGK..IGL, $\beta = -0.53$, permutation $p = .005$, FDR $= 0.50$).

TABLE 1 Demographic and lifestyle characteristics of workers exposed to diesel engine exhaust and controls

Characteristic	Controls ($n = 19$)	Exposed ($n = 19$)	*p* Value
Age in years, mean (*SD*)	44.6 (6.7)	41.7 (6.5)	.18[a]
Body mass index (kg/m^2), mean (*SD*)	25.3 (4.4)	25.1 (3.2)	.86[a]
Elemental carbon (μg/m^3), median (range)[b]	0.0 (0.0)	68.2 (40.2–107.7))	<.001[a]
Current smoker, *n* (%)			
No	3 (15.8)	4 (21.1)	
Yes	16 (84.2)	15 (78.9)	1.00[c]
Current alcohol use, *n* (%)			
No	2 (10.5)	4 (21.1)	
Yes	17 (89.5)	15 (78.9)	.66[c]
Recent infection (%)			
No	10 (52.6)	6 (31.6)	
Yes	9 (47.4)	13 (68.4)	.32[c]

Abbreviations: DEE, diesel engine exhaust; EC, elemental carbon; *SD*, standard deviation.
[a]*p* Value of *t*-test.
[b]Background adjusted EC concentrations. Based on detailed walk-through surveys, no DEE sources were identified in control factories. Hence, EC exposure levels in control factories were used as a proxy for background outdoor exposure in this region to adjust for exposure in all participants.
[c]*p* Value of Fisher's exact test.

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TABLE 2 Mean (*SD*) serum level of selected SOMAscan proteins (relative fluorescence unit) in controls and workers exposed to diesel engine exhaust, and its surrogate, elemental carbon (below and above the median)

		DEE exposure (yes, no)						Levels of EC exposure			
Proteins	Description	Unexposed ($n = 19$)	Exposed ($n = 19$)	% mean difference	β[a]	p Value	Permutation p value[b]	Low exposed ($n = 9$)	High exposed ($n = 10$)	p trend	Permutation p trend[b]
CXCL11	Chemokine (C-X-C motif) ligand 11	4121.1 (2035.8)	2326.2 (696.1)	−43.6	−0.25	.00003	.00004	2356.7 (720.8)	2298.8 (710.9)	.0005	.0005
HAPLN1	Hyaluronan and proteoglycan link protein1	1338.3 (417.6)	958.7 (220.0)	−28.4	−0.11	.0017	.0012	967.1 (240)	951 (213.3)	.0032	.0051
FLT4	FMS-related tyrosine kinase 4	10953.5 (2277.6)	8654.1 (1632.8)	−21.0	−0.10	.0022	.0024	8867.3 (1914.2)	8462.2 (1410.2)	.0050	.0064
CD40LG	CD40 ligand	557.9 (271.1)	363.7 (233.6)	−34.8	−0.21	.0029	.0027	353.2 (152.2)	373.1 (297.2)	.0099	.0072
PES1	Pescadillo ribosomal biogenesis factor 1	387.5 (119.5)	296.4 (139.2)	−23.5	−0.16	.0046	.0035	322.3 (142)	273.1 (139.8)	.0043	.0046
SHH	Sonic hedgehog	1112.7 (506.4)	722.3 (201.3)	−35.1	−0.15	.0049	.0041	727.4 (205.7)	717.7 (208.2)	.0115	.0156
CCL5	Chemokine (C-C motif) ligand 5	64683.3 (25291.4)	41808.9 (17169.9)	−35.4	−0.18	.0083	.0044	39992.9 (17993.1)	43443.3 (17191.9)	.0294	.0177
P4HB	Prolyl 4-hydroxylase, beta polypeptide	370.2 (121.7)	264.6 (50.2)	−28.5	−0.12	.0048	.0045	264.9 (55.7)	264.3 (47.8)	.0105	.0117
CCL20	Chemokine (C-C motif) ligand 20	349.4 (106.2)	263.2 (112.9)	−24.7	−0.14	.0059	.0047	270.8 (112.4)	256.3 (118.9)	.0150	.0175
IGHE. IGK.. IGL	Immunoglobulin heavy constant epsilon; immunoglobulin kappa locus; immunoglobulin lambda locus	17555.2 (21420.6)	5577.6 (6591.3)	−68.2	−0.53	.0070	.0049	5817.7 (7917.5)	5361.5 (5573.2)	.0101	.0095
TNFSF9	Tumor necrosis factor (ligand) superfamily 9	419.5 (144.7)	321.3 (86.2)	−23.4	−0.11	.0059	.0053	339.3 (75.9)	305 (95.5)	.0055	.0083
PGD	Phosphogluconate dehydrogenase	3163.8 (1931.8)	1945.5 (833.6)	−38.5	−0.22	.0047	.0054	2158.6 (716)	1753.7 (920.7)	.0039	.0022
PPID	Peptidylprolyl isomerase D	505.7 (365.7)	332.7 (161.5)	−34.2	−0.17	.0096	.0058	363.1 (167.4)	305.5 (159.7)	.0133	.0110
ANGPT2	Angiopoietin 2	144.6 (31.6)	116.6 (23.6)	−19.4	−0.07	.0069	.0063	118.3 (29.9)	115 (17.9)	.0095	.0199
PPY	Pancreatic polypeptide	7204.6 (3671.1)	4063.1 (1516.3)	−43.6	−0.19	.0075	.0064	3554.5 (1060.1)	4520.9 (1762.8)	.0466	.0546
SPINT1	Serine peptidase inhibitor, Kunitz type 1	6863.5 (1411.0)	8164.2 (1743.4)	19.0	0.08	.0066	.0065	8439.2 (1755.1)	7916.7 (1788.1)	.0327	.0297

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TABLE 2 (Continued)

		DEE exposure (yes, no)						Levels of EC exposure			
Proteins	Description	Unexposed ($n = 19$)	Exposed ($n = 19$)	% mean difference	β[a]	p Value	Permutation p value[b]	Low exposed ($n = 9$)	High exposed ($n = 10$)	p trend	Permutation p trend[b]
PLA2G1B	Phospholipase A2, group IB	974.4 (192.5)	1427.8 (707.6)	46.5	0.13	.0081	.0070	1282.5 (375.2)	1558.6 (914.4)	.0099	.0057
NAGK	*N*-acetylglucosamine kinase	683.8 (262.0)	504.8 (143.3)	−26.2	−0.12	.0082	.0079	589.3 (106.8)	428.7 (131.7)	.0006	.0003
CA3	Carbonic anhydrase III, muscle specific	1394.9 (642.6)	1779.8 (857.0)	27.6	0.13	.0058	.0079	1929.1 (482.7)	1645.4 (1104.2)	.0825	.1020
CCL25	Chemokine (C-C motif) ligand 25	2918.9 (1733.9)	1863.8 (826.5)	−36.1	−0.18	.0071	.0083	1986.6 (682.1)	1753.1 (961.2)	.0054	.0034
CCL4L1	C-C Motif Chemokine Ligand 4 Like 1	2447.5 (1158.6)	1662.9 (896.6)	−32.1	−0.20	.0091	.0083	1902.3 (820.1)	1447.4 (949.2)	.0052	.0059
PDXK	Pyridoxal (pyridoxine, vitamin B6) kinase	2544.2 (1162.0)	1910.0 (595.9)	−24.9	−0.11	.0071	.0084	2091.9 (654)	1746.2 (516.7)	.0079	.0073

Abbreviations: DEE, diesel engine exhaust; EC, elemental carbon; *SD*, standard deviation.
[a]Linear regression models adjusted for age, current smoking, and body mass index.
[b]Permutation p values calculated based on 500,000 iterations.

3.3 | Relationship between DEE-related serum proteins and level of EC

Of 22 DEE-related proteins, 13 proteins (CXCL11, HAPLN1, FLT4, CD40LG, PES1, IGHE.IGK..IGL, TNFSF9, PGD, NAGK, CCL25, CCL4L1, PDXK, and PLA2G1B) showed a significant exposure-response relationship with EC (p trend < .01) (Table 2). Of these, serum levels of all but PLA2G1B were declining with increasing air levels of EC (Table 2 and Figure 1). For instance, the mean serum level of CXCL11 among unexposed controls was 4121.1 RFU compared to 2356.7 RFU among the lower exposed workers (EC air level, range: 40.2–62.1 μg/m^3) and 2298.8 RFU among the higher exposed workers (EC air level, range: 66.9–107.7 μg/m^3; p trend $=$ .0005). Apart from these 13 proteins, four proteins (FGF2, A2M, ENO1, and NGF) also showed significant exposure-response relationships with EC, where serum levels of all but NGF were declining with increasing air levels of EC. (Table S1).

3.4 | System-level molecular response to DEE

To understand the system-level molecular response to DEE exposure, we constructed correlation-based networks of 22 DEE-related proteins, separately for the exposed and unexposed groups. While we observed weak to moderate correlations for certain proteins among the controls, a distinctively different and stronger correlation pattern was observed among exposed workers (Figure 2). For instance, PES1, CD40LG, CCL20, PDXK, CCL5, CCL4L1, and TNFSF9 showed moderate to strong positive correlations (Spearman's $\rho = 0.55$–0.92; $p = 7.5e{-}03$–$2.8e{-}09$) among the exposed workers compared to the controls (Spearman's $\rho = 0.03$–0.61; $p = 9.0e{-}01$–$5.3e{-}03$) (Table S2).

3.5 | Biological response to DEE exposure

Pathway analysis of DEE-related proteins using MetaCore (Thomson Reuters; https://portal.genego.com/) revealed significant enrichment (FDR < 0.05) for several immune and inflammatory pathways (Table 3). Specifically, two or more of the seven proteins (PES1, CD40LG, CCL20, PDXK, CCL5, CCL4L1, and TNFSF9) that showed distinctively different and stronger correlation patterns among DEE exposed workers showed significant enrichment for Th17 cell migration, immune response to T-cell subsets, generation of cytotoxic CD8+ T cells in COPD, and immune response involving IL-4-responsive genes in type 2 immunity. DEE-related proteins also showed enrichment for endothelial differentiation during embryonic development, IFN-gamma and Th2 cytokines-induced inflammatory signaling, and PDGF signaling via JAK–STAT and ROS (Table 3). These pathways were also among the top-identified pathways when we included 126 proteins based on their associations with DEE at the level of permutation p < .05 (Table S3).

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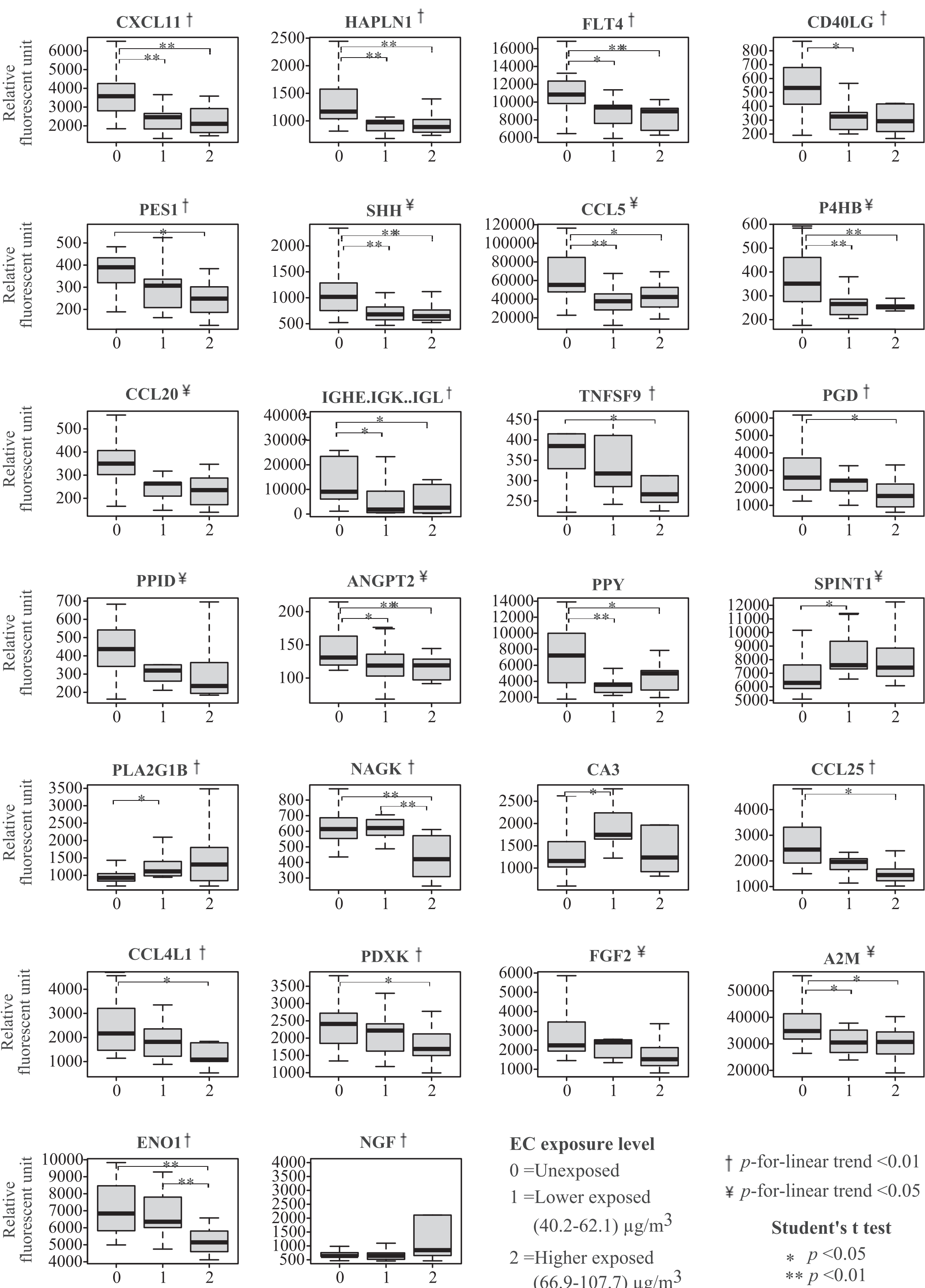

FIGURE 1 Box and whisker plots showing mean (95% confidence intervals) serum levels (relative fluorescence unit) of selected proteins that showed a significant difference in concentrations between workers exposed to diesel engine exhaust and unexposed controls by levels of elemental carbon air level (0 = unexposed controls; 1 = exposed below the median concentration, 66.9 μg/m^3; range: 40.2–62.1 μg/m^3; 2 = exposed above the median, range: 66.9–107.7 μg/m^3). *p* values for linear trend test (EC levels treated as 0, 1, 2 ordinal variables) are indicated as $^{†}p < .01$; $^{¥}p < .05$. Differences in mean protein levels between EC categories were tested using Student's t-test; *p* values are indicated as: *$p < .05$; **$p < .01$

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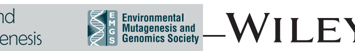

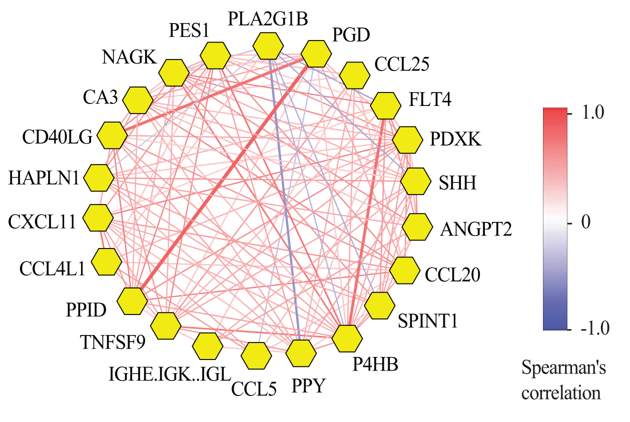

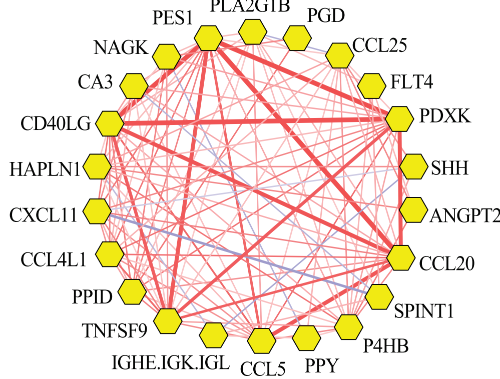

FIGURE 2 Correlation-based networks of DEE-related serum proteins among (a) exposed and (b) unexposed workers. Networks were constructed based on Spearman's rank correlation coefficients of all possible pairs of DEE-related serum proteins with an effect size of Spearman's $|\rho| \geq 0.25$ using Cytoscape. The nodes represent serum proteins, and the edges represent correlations between proteins. The color gradient represents the direction and strength of correlation, whereas the thickness of the connection represents the strength of correlation (correlation coefficients are presented in Table S2). DEE, diesel engine exhaust

TABLE 3 Summary of enrichment analysis by curated pathway maps between the control and diesel exposure groups

Pathway maps	Proteins involved in the pathway[a]	FDR[b]
Common mechanisms of Th17 cell migration	CXCL11/I-TAC, CCL25, CCL5, CCL20, MIP-1-beta/CCL4L1	3.2E−06
Immune response to T cell subsets: secreted signals	CCL5, CCL20, MIP-1-beta/CCL4L1	6.6E−04
Endothelial differentiation during embryonic development	SHH, VEGFR-3/FLT-4, ANGPT2	2.9E−03
Generation of cytotoxic CD8+ T cells in COPD	CD40LG(TNFSF5), TNFSF9 (CD137)	2.2E−02
IFN-gamma and Th2 cytokines-induced inflammatory signaling in normal and asthmatic airway epithelium	CXCL11/I-TAC, CCL5	2.7E−02
Chemotaxis: CCL16-, CCL20-, CXCL16- and CCL25-mediated cell migration	CCL25, CCL20	2.9E−02
TNF-alpha and IL-1 beta-mediated regulation of contraction and secretion of inflammatory factors in normal and asthmatic airway smooth muscle	CCL5, PLA2G1B	3.8E−02
Signal transduction: PDGF signaling via JAK–STAT and reactive oxygen species (ROS)	CXCL11/I-TAC, CCL5	3.8E−02
Immune response: IL-4-responsive genes in type 2 immunity	CD40LG(TNFSF5), CCL5	4.0E−02

Note: Proteins that showed stronger intercorrelations among exposed workers compared to unexposed are bold faced.
Abbreviation: FDR, false discovery rate.
[a]Twenty-two proteins that were associated with diesel engine exhaust (DEE) at the level of permutation $p < .01$ were included in pathway analysis. Pathway analysis of 126 proteins based on their associations with DEE at permutation $p < .05$ were presented in Table S3.
[b]Pathways were considered noteworthy if FDR < 0.05.

4 | DISCUSSION

In this cross-sectional molecular epidemiological study, we compared serum levels between DEE exposed and unexposed workers for 1317 proteins quantified using a targeted proteomics platform (SOMALogic). We found that occupational exposure to DEE was associated with altered levels of 22 proteins; 13 of which (CXCL11, HAPLN1, FLT4, CD40LG, PES1, IGHE.IGK..IGL, TNFSF9, PGD, NAGK, CCL25, CCL4L1, PDXK, and PLA2G1B) showed a significant exposure-response relationship with EC, with serum levels of all but PLA2G1B declining with increasing air levels of EC. These proteins showed distinctive correlation patterns by DEE exposure status. For instance, PES1, CD40LG, CCL20, PDXK, CCL5, CCL4L1, and TNFSF9 were highly correlated among exposed workers but not among controls, suggesting that these DEE-related proteins may be involved in common biological pathways. Pathway analysis showed that these distinctively correlated proteins were enriched in inflammation and immune regulation pathways, providing insights into the mechanisms of DEE-induced carcinogenicity.

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Of 22 DEE-related serum proteins, six were chemokines (CXCL11, CCL5, CCL20, CCL25) and immune cell surface markers (CD40LG, TNFSF9), which play an important role in inflammation and immune regulation. The strongest association was observed for C-X-C Motif Chemokine Ligand 11 (CXCL11), also known as T-cell alpha chemoattractant (I-TAC), which is a chemokine of the CXC chemokine family. CXCL11/I-TAC also belongs to a group of IFN-inducible chemokines, which mediate the recruitment of T cell, natural killer cell, and monocyte/macrophage, mainly through their cognate G-protein coupled receptor, CXCR3 (Janatpour et al., 2001). We observed a negative association for CXCL11/I-TAC with DEE exposure, which is consistent with a previous study in the same population despite CXCL11/I-TAC was measured using a targeted Luminex bead-based assay in the plasma (Bassig et al., 2017), suggesting that our results are internally valid. In addition, in vitro studies showed that higher exposure to DEE was associated with lower concentrations of several chemokines, including CXCL11/I-TAC in the human macrophage (Jaguin et al., 2015) as well as suppression of macrophage function and pulmonary clearance (Yang et al., 2001). Taken together, these findings suggest that chronic DEE exposure could potentially lead to sustained suppression of alveolar macrophage function and pulmonary clearance, resulting in chronic airway inflammation, a process increasingly being recognized as an important contributor to lung cancer (Shiels et al., 2011).

The exact mechanisms of how different DEE-related serum proteins may increase the risk of carcinogenicity are yet to be elucidated, although prior studies suggest that inflammation and immune regulation may play a key role. For example, CD40LG is a transmembrane cytokine and surface marker of T cells that acts as a ligand to CD40/TNFSF5 to regulate B cell function (Li et al., 2013; Wang et al., 2019). TNFSF9 is a transmembrane cytokine expressed in the carcinoma cell lines and acts as a ligand for TNFRSF9/4-1BB to interact between tumor and T cells (Dostert et al., 2019). Chemokines such as CCL5 (C-C Motif Chemokine Ligand 5), CCL20 (C-C Motif Chemokine Ligand 20, also known as macrophage inflammatory protein-3a), and CCL4L1 (C-C Motif Chemokine Ligand 4 Like 1, also known as macrophage inflammatory protein-1-beta) are involved in the migration of blood monocytes, memory T-helper cells, and other inflammatory cells. CCL20 is also involved in T-cell recruitment (Homey et al., 2000) and pathogenesis of interstitial lung fibrosis and chronic obstructive pulmonary disease (COPD) (Bracke et al., 2007; Demedts et al., 2007), chronic lung conditions considered as risk factors for lung cancer (Choi et al., 2018; Durham & Adcock, 2015; Naccache et al., 2018; Sekine et al., 2012). However, our observed negative associations for a few proinflammatory markers (i.e., CCL5, CCL20, CCL4L1) with DEE exposure require further evaluation. It is possible that some markers or pathways involved in lung carcinogenesis may be distinct in the setting of high DEE exposure versus those involved in a scenario without identified high exposure to this known risk factor. It is also possible that some of the markers identified in this study may not reflect a direct consequence of DEE exposure rather an adaptive response to mitigate the effect of other DEE-related proinflammatory/immune markers.

We observed a distinct correlation pattern of proteins between DEE exposed and unexposed workers. Specifically, serum levels of PES1, CD40LG, CCL20, PDXK, CCL5, CCL4L1, and TNFSF9 were highly correlated among DEE exposed workers (Spearman's $\rho = 0.55$–0.92; $p = 7.5e{-03}$–$2.8e{-09}$) but not among controls (Spearman's $\rho = 0.03$–0.61; $p = 5.3e{-03}$–$9.0e{-01}$). These findings suggest that DEE exposure may be related to biological perturbations involving these proteins in common molecular pathways or different pathways but biochemically they change in the same direction. Pathway enrichment analysis revealed that these distinctively correlated proteins were enriched in inflammation and immune regulation pathways, such as common mechanisms of Th17 cell migration, immune response to T cell subsets, generation of cytotoxic CD8+ T cells in COPD, and immune response involving IL-4-responsive genes in type 2 immunity. Interestingly, we showed in a previous study that DEE exposure was associated with the absolute count of total lymphocyte and lymphocyte subsets (CD4+ T cells, CD8+ T cells, and B cells) irrespective of the worker's smoking status (Lan et al., 2015). These findings add to the evidence that inflammation and immune regulation may play an important role in DEE-induced carcinogenicity.

The primary limitation of our study was the relatively small sample size, which increases the probability of false-positive findings given the high number of proteins being assessed (Wacholder et al., 2004). As such, these findings should be viewed as exploratory and will require further exploration in larger studies. In addition, our findings need to be evaluated in light of residual confounding by unmeasured confounders such as nutritional and socioeconomic statuses and traffic-related exposures. Exposure to EC in diesel factory workers (median [range] = 68.2 [40.2–107.7] $\mu g/m^3$) in this study was much higher than the occupational exposure limit (OEL) in many populations. Hence, our findings may not be generalizable to other populations with different exposure distributions. For example, the European Union has adopted the OEL of 50 $\mu g/m^3$ for EC, set to take effect from 2023 in general industries (Le parlement Europeen et le conseil de l'union Europeenne, 2019) while the Netherlands has adopted a much lower OEL for EC of 10 $\mu g/m^3$ (Health Council of the Netherlands, 2019). There is no national standard for EC in the USA, although the American Conference of Governmental Industrial Hygienists proposed a threshold limit value of 20 $\mu g/m^3$ for EC and the U.S. Mining Safety and Health Administration set the criteria for total carbon at 160 $\mu g/m^3$ (EC conversion factors range between 1.19 and 1.44) (Birch, 2003; Noll et al., 2015). Despite these limitations, identification of markers and pathways related to inflammation and immune regulation, as prior studies have suggested (Chiang et al., 2013; Shiels et al., 2011), indicate the biological relevance of our findings. Specifically, we observed suggestive negative associations with DEE for IL-10 ($\beta = -0.08$, permutation $p = .08$) and IL-1B ($\beta = -0.11$, permutation $p = .03$), markers that were negatively associated with lung cancer among never-smoking women in the Shanghai Women's Health Study (Shiels et al., 2017). Other strengths of our study include the availability of EC exposure data measured using personal monitors, which enabled us to evaluate the exposure-response relationship between serum proteins and levels of EC.

In summary, we measured serum levels of 1317 proteins in a cross-sectional molecular epidemiological study of occupational DEE

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exposure in China. We identified 22 serum proteins associated with DEE exposure. These proteins were significantly enriched in several immunoregulatory and inflammatory pathways, providing insight into the mechanisms of DEE-induced carcinogenesis. Given the small sample size of this study, we encourage to confirm our findings in larger studies.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Qing Lan, Nathaniel Rothman, Debra T. Silverman, Yuxin Zheng, Roel Vermeulen, and Bryan A. Bassig designed the study, and supervised data collection. Mohammad L. Rahman prepared the analysis plan, analyzed data, and wrote the manuscript. Yufei Dai, Wei Hu, Huawei Duan, Yong Niu, Jun Xu, Wei Fu, Baosen Zhou, Meng Ye, Xiaowei Jia, Tao Meng, Ping Bin contributed to data and biological sample collection. All authors contributed to the interpretation of the results and revision of the manuscript for important intellectual content and approved the final version of the manuscript. Mohammad L. Rahman, Qing Lan, and Nathaniel Rothman are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online supplementary material.

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