Original research

Alterations to biomarkers related to long-term exposure to diesel exhaust at concentrations below occupational exposure limits in the European Union and the USA

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ABSTRACT

Background We previously found that occupational exposure to diesel engine exhaust (DEE) was associated with alterations to 19 biomarkers that potentially reflect the mechanisms of carcinogenesis. Whether DEE is associated with biological alterations at concentrations under existing or recommended occupational exposure limits (OELs) is unclear.

Methods In a cross-sectional study of 54 factory workers exposed long-term to DEE and 55 unexposed controls, we reanalysed the 19 previously identified biomarkers. Multivariable linear regression was used to compare biomarker levels between DEE-exposed versus unexposed subjects and to assess elemental carbon (EC) exposure-response relationships, adjusted for age and smoking status. We analysed each biomarker at EC concentrations below the US Mine Safety and Health Administration (MSHA) OEL (<106 µg/m^3), below the European Union (EU) OEL (<50 µg/m^3) and below the American Conference of Governmental Industrial Hygienists (ACGIH) recommendation (<20 µg/m^3).

Results Below the MSHA OEL, 17 biomarkers were altered between DEE-exposed workers and unexposed controls. Below the EU OEL, DEE-exposed workers had elevated lymphocytes (p=9E-03, false discovery rate (FDR)=0.04), CD4+ count (p=0.02, FDR=0.05), CD8+ count (p=5E-03, FDR=0.03) and miR-92a-3p (p=0.02, FDR=0.05), and nasal turbinate gene expression (first principal component: p=1E-06, FDR=2E-05), as well as decreased C-reactive protein (p=0.02, FDR=0.05), macrophage inflammatory protein-1β (p=0.04, FDR=0.09), miR-423-3p (p=0.04, FDR=0.09) and miR-122-5p (p=2E-03, FDR=0.02). Even at EC concentrations under the ACGIH recommendation, we found some evidence of exposure-response relationships for miR-423-3p (p_{trend}=0.01, FDR=0.19) and gene expression (p_{trend}=0.02, FDR=0.19).

Conclusions DEE exposure under existing or recommended OELs may be associated with biomarkers reflective of cancer-related processes, including inflammatory/immune response.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Diesel engine exhaust (DEE) is a known carcinogen and was previously found to be associated with alterations to various biomarkers, some of which are related to lung cancer risk.

⇒ Whether long-term exposure to DEE is associated with early biological changes at concentrations below existing or recommended occupational exposure limits (OEL) in the European Union (EU) and the USA is unclear.

WHAT THIS STUDY ADDS

⇒ Even below the EU OEL of 50 µg/m^3 of elemental carbon, we found evidence that long-term DEE exposure is associated with alterations to some biomarkers related to lung cancer.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings support more stringent OELs in the EU and the USA to protect the respiratory health of DEE-exposed workers.

INTRODUCTION

Diesel engine exhaust (DEE) contains genotoxic constituents such as nitrated polycyclic aromatic hydrocarbons as well as fine particulate matter 2.5 and has been designated as a group 1 human carcinogen by the International Agency for Research on Cancer.[1] The constituents of DEE may contribute to cancer development by inducing oxidative stress, inflammation[2 3] and DNA damage[4]; however, the underlying mechanisms of action remain unclear. To investigate the potential carcinogenic mechanisms of long-term exposure to DEE, we conducted a cross-sectional molecular epidemiology study of workers in a diesel engine manufacturing facility and unexposed controls, including biospecimen

collection and air monitoring.[3] Notably, the diesel engine factory workers had a wide range of exposure to DEE (elemental carbon (EC) 6.1–107.7 μg/m^3),[5] which even at the lower range, was comparable to some urban environments (maximum daily EC 1.2–16 μg/m^3).[6]

Markers of early biological effect are intermediate biomarkers that generally measure changes that reflect early and often non-persistent effects.[7] Early biological effects relevant to exposures and future disease risk occur during some aetiological time window before biological effects that occur later on that are the result of underlying predisease (ie, subclinical disease), disease onset or disease progression. In previous analyses,[2 3 5 8–10] occupational exposure to DEE was found to be associated with alterations to biomarkers that may reflect the key characteristics of carcinogens.[11] In particular, we found alterations to lymphocyte subsets that reflect inflammatory states, cytokines/chemokines that reflect immune function, arthrobacter luteus (Alu) retroelement copy number that reflects genomic instability, urinary mutagenicity that reflects recent exposure to genotoxic agents, circulating microRNAs (miRNAs) involved in post-transcriptional regulation[12] and gene expression in nasal epithelial cells.[2 3 5 8–10 13] Although we observed significant trends for many biomarkers, we did not determine if levels of most of these biomarkers were altered at DEE concentrations below occupational exposure limits (OELs) that have been adopted or recommended in the USA or Europe.

The extensive use of diesel engines in transportation and industrial settings exposes millions of workers around the world to DEE. OELs for EC, a surrogate measure of DEE, differ by country and industry. In the USA, the Mine Safety and Health Administration (MSHA) has the OEL for diesel particulate matter currently set at 160 μg/m^3 of total carbon (equivalent to 106 μg/m^3 of EC.[14] The European Union (EU) adopted a more stringent OEL of 50 μg/m^3 for EC in December 2018, which will begin in 2026 for underground mines and tunnel construction and in 2023 for other industries.[15] Although intended to protect the respiratory health of DEE-exposed workers, concerns remain over the new EU OEL, as it potentially leaves a considerable risk of DEE-related cancers. Indeed, we previously found evidence of a biological effect of DEE on urinary mutagenicity, a biomarker that reflects recent exposure to genotoxic pollutants, even below the EU OEL of 50 μg/m^3 for EC.[13] The American Conference of Governmental Industrial Hygienists (ACGIH) recommends an even lower workplace exposure limit of 20 μg/m^3 to protect the health of exposed workers.[16]

To further assess the early biological effects of long-term exposure to DEE at relatively lower levels, we conducted new analyses on previously identified DEE-related biomarkers at EC concentrations below the MSHA OEL of 106 μg/m^3, below the new EU OEL of 50 μg/m^3 and below the ACGIH recommended workplace exposure limit of 20 μg/m^3. Findings from our study could potentially provide mechanistic evidence of the harmful effects of DEE, even at levels below current, proposed or recommended regulatory limits, and reinforce the need for more stringent OELs, such as those recently adopted by the Netherlands at 10 μg/m^3 of EC.[15]

METHODS

Cross-sectional molecular epidemiology study

Our cross-sectional molecular epidemiology study, including the molecular assays used to measure various biomarkers, has been described in detail.[2 3 9 13] The demographic, anthropometric, lifestyle and exposure characteristics of the overall study population are shown in online supplemental table 1. Briefly, we conducted an occupational study of 54 male workers who were exposed long-term to DEE in the workplace and a comparison group of 55 male unexposed controls in China in March 2013. DEE-exposed workers were selected from an engine testing facility of a factory located in northeastern China that manufactures diesel engines for light and heavy trucks. The DEE-exposed workers were enrolled from a pool of engine testers that were identified to have the longest periods of employment and were either never-smokers or light-smokers. The 55 unexposed controls were recruited from local workplaces that did not use diesel equipment or have processes that resulted in exposure to any known or suspected genotoxic, haematotoxic or immunotoxic chemicals or above-background particulate levels based on assessments from detailed walkthrough surveys.[3] The control workplaces included a brewery (n=24), a water treatment plant (n=18), a meat packing facility (n=8) and an administrative facility (n=5) from the same city as the diesel engine factory. Given that the diesel factory workers and unexposed controls were sampled from the same local region, their environmental exposure (outside of the workplace) to diesel exhaust and outdoor air pollution were expected to be similar. The unexposed participants were frequency-matched to DEE-exposed workers by age (±5 years) and smoking status (ie, never, former, current).

From October 2012 to March 2013, the exposure assessment was conducted in the diesel engine testing facility. We collected repeated full-shift personal air samples for EC, a surrogate measurement for DEE exposure[17] and other constituents.[3] EC was measured on the quartz filters using NIOSH Method 5040.[18] Weights were divided by the volume of air drawn through the filters to provide exposure concentrations (μg/m^3). Individual exposure levels were estimated using mixed effects models.[3] Biospecimen collection and measurement procedures of the various biomarkers are described in the online supplemental appendix 1. Briefly, peripheral blood samples were collected after air sampling for complete blood cell count, analysis of the major lymphocyte subsets via flow cytometry (FACSCalibur, BD Bioscience),[3] assessment of inflammatory/immunological cytokines/chemokines,[2 19] measurement of serum miRNA concentrations[12] and extraction of leucocyte genomic DNA for quantitative PCR measurement of Alu retroelement copy number.[5] Overnight urine samples were collected following the participants' work shift and urinary isolates were processed and enzymatically deconjugated for measurement of urinary mutagenicity using the Ames test.[13] Additionally, we collected nasal turbinate epithelial cell samples for RNA extraction and transcriptomics analyses with Affymetrix Human Gene 1.0 ST GeneChips (Affymetrix, Santa Clara, California, USA) as previously described.[10]

Statistical analyses

In the current study, we performed new analyses on 17 biomarkers from circulating whole blood, leucocytes, plasma or serum that were previously found to be associated with DEE (exposed vs unexposed) at $p<0.05$ or had an EC exposure-response relationship with $p_{trend}<0.05$[2 3 10 13 19 12](table 1). These circulating biomarkers potentially reflect the early biological effects of long-term exposure to DEE after some of its constituents diffuse from the alveoli into the bloodstream. Additionally, we analysed the first principal component (PC1) of 225 diesel signature genes from a previously reported transcriptomics analysis of nasal turbinate epithelial cell samples, which potentially reflects the chronic localised effect of both organic and particulate phases

Table 1 Biomarkers previously found to be associated with DEE

Study	Biomarker, unit	Reported effect size, % difference, DEE-exposed versus unexposed	P value, DEE versus unexposed, adjusted	EC p_{trend}, adjusted, all available categories and subjects with data
Lan *et al*[3]	Total lymphocyte count, cells/µL	+15.5	4E-04	2E-04
	CD4+ T cells, cells/µL	+24.6	2E-04	3E-05
	CD8+ T cells, cells/µL	+17.3	0.01	0.02
	B cells, cells/µL	+20.5	0.02	0.01
Bassig *et al*[2]	CRP, pg/mL	−42.7	0.01	0.05
	CCL15/MIP-1D, pg/mL	+21.2	0.02	0.01
	CXCL11/ITAC, pg/mL	−19.3	0.03	0.04
	IL-16, pg/mL	+16.6	0.02	0.06
	sILRII, pg/mL	+8.00	0.08	0.03
	SGP130, pg/mL	+6.60	0.13	0.05
Dai *et al*[19]	MIP-1β, pg/mL	−37.1	<0.001	N/A
Drizik *et al*[10]	Summarised RNA expression from 225 DEE signature genes in nasal epithelial cells into a single value using the first PC	Higher among DEE-exposed subjects	8.11E−11	<0.0001
Wong *et al*[5]	Alu retroelement copy number	+3.80	0.03	0.02
Wong *et al*.[13]	Urinary mutagenicity	+132.1	0.02	2E-04
Hu *et al*[12]	miR-191-5p, AU	−23.00	0.002	0.001
	miR-93-5p, AU	−7.00	0.03	0.01
	miR-423-3p, AU	−25.00	0.02	0.05
	miR-122-5p, AU	−43.00	0.01	0.10
	miR-92a-3p, AU	+6.00	0.05	0.30

Alb, albumin; Alu, arthrobacter luteus; AU, arbitrary units for microRNAs; CD, cluster of differentiation; CRP, C reactive protein; CXCL, chemokine (C-X-C motif) ligand; DEE, diesel engine exhaust; EC, elemental carbon; IL, interleukin; ITAC, interferon-inducible T cell alpha chemoattractant; MIP, macrophage inflammatory protein; miR, microRNA; PC, principal component; sILR, soluble interleukin receptor.

of DEE on directly exposed tissue.[10] We also analysed urinary mutagenicity, a biomarker reflective of recent exposure to genotoxic agents, from a subset of participants with sufficient amounts of urine.[13] Data from a recent proteomic study of 19 DEE-exposed and 19 unexposed subjects were not included in this new analysis because the exposed subjects were selected to have higher DEE concentrations (mostly >50 µg/m^3).[9]

We conducted three separate sets of analyses on each of the selected biomarkers. The first analyses were restricted to unexposed controls and subjects exposed to EC concentrations below the MSHA OEL of 106 µg/m^3 (equivalent to 160 µg/m^3 total carbon).[2 3 5 19] The second analyses were restricted to unexposed controls and subjects exposed to EC concentrations below the EU OEL of 50 µg/m^3. The third analyses were restricted to unexposed controls and subjects exposed to EC concentrations below 20 µg/m^3, which is the recommended limit of the ACGIH and has been adopted by the State of California, Department of Health Services as the OEL.[20] We estimated differences in continuous natural log-transformed levels of each circulating biomarker between DEE-exposed and unexposed subjects using multivariable linear regression models that were parsimoniously adjusted for age and smoking status (never, former, current) to avoid overfitting. We considered adjusting for educational attainment; however, its inclusion did not qualitatively change the results and was thus excluded from the final analyses. Urinary mutagenicity analyses were adjusted for age and never versus former smoking because there were no current smokers in those analyses. Additionally, we estimated exposure-response relationships between continuous EC (µg/m^3) and continuous natural log-transformed levels of each circulating biomarker. PC1 of the gene expression signature and urinary mutagenicity were not natural log-transformed. The 17 circulating biomarkers, gene expression signature and urinary mutagenicity were selected a priori from previous studies for statistically significant associations with DEE exposure ($p<0.05$ or $p_{trend}<0.05$) across the full range of EC concentrations. In the current analyses, we calculated the p values and Benjamini-Hochberg false discovery rate (FDR) q values. Biomarkers with both $p<0.05$ and FDR<0.20 for DEE-exposed versus unexposed at EC<106 µg/m^3, and at either EC<50 µg/m^3 or EC<20 µg/m^3 were considered noteworthy. In separate parallel analyses, biomarkers with a $p_{trend}<0.05$ and FDR<0.20 for continuous trend were considered noteworthy. We assessed the correlation structure among the biomarkers using Spearman's rank correlations.

RESULTS

Figure 1 shows the distributions of the 17 selected circulating biomarkers and nasal epithelium gene expression signature among the unexposed controls and DEE-exposed workers at EC<106 µg/m^3, EC<50 µg/m^3 and EC<20 µg/m^3. Under the MSHA limit of 106 µg/m^3, 15 of the selected circulating biomarkers differed by DEE-exposure status at $p<0.05$ and FDR<0.20, while 13 had evidence of an exposure-response relationship at $p_{trend}<0.05$ and FDR<0.20 (table 2). Among these signals, eight circulating biomarkers differed between DEE-exposed workers and unexposed controls at EC concentrations below the EU OEL (figure 1, table 2). At EC<50 µg/m^3, DEE-exposed workers had elevated lymphocytes (p=9E-03, FDR=0.04), CD4+ count (p=0.02, FDR=0.05), CD8+ count (p=5E-03, FDR=0.03) and miR-92a-3p (p=0.02, FDR=0.05), as well as decreased CRP (p=0.02, FDR=0.05), MIP-1β (p=0.04, FDR=0.09), miR-423-3p (p=0.04, FDR=0.09) and miR-122-5p (p=2E-03, FDR=0.02) compared with unexposed controls (figure 1, table 2). Among these findings, lymphocytes, CD8+ count, CRP, miR-423-3p, miR-122-5p and miR-92a-3p had noteworthy exposure-response relationships with EC (table 2). We did not find consistent and noteworthy evidence

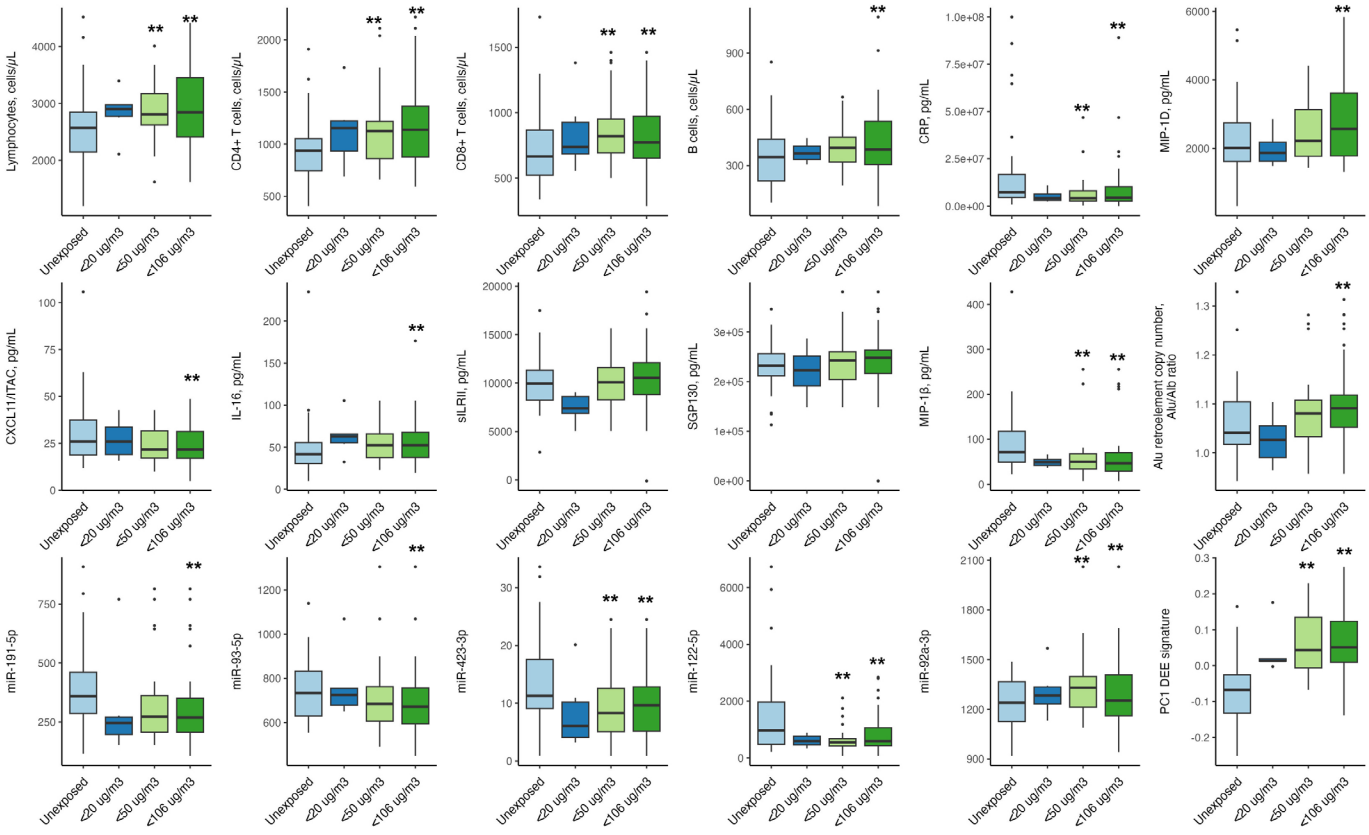

Figure 1 Alterations to selected biomarkers when comparing diesel engine exhaust (DEE)-exposed workers with unexposed controls at elemental carbon (EC) concentrations below the US Mine Safety and Health Administration occupational exposure limit (OEL) (106 µg/m^3), the European Union OEL (50 µg/m^3) and the American Conference of Governmental Industrial Hygienists recommendation (20 µg/m^3). The three analyses comparing DEE-exposed with unexposed controls were not independent of each other. The DEE-exposed subjects at each EC concentration included DEE-exposed subjects from the lower exposure categories. **P<0.05 and FDR<0.20 for the comparison of DEE-exposed subjects with EC concentration below the OEL versus unexposed controls. The specific p values and FDR q values are presented in table 2. A graph for urinary mutagenicity is not shown because similar data have been reported in a previous publication (Wong *et al*[13]). CRP, C reactive protein; CXCL, chemokine (C-X-C motif) ligand; IL, interleukin; MIP, macrophage inflammatory protein; miR, microRNA; PC, principal component; sILR, soluble interleukin receptor.

for biomarker alterations by DEE exposure status below the ACGIH recommended limit of EC<20 µg/m^3. However, there were some indications of noteworthy EC exposure-response relationships for IL-16 and miR-423-3p in spite of limited and unbalanced data at EC<20 µg/m^3 (table 2). There was modest correlation between miR-423-3p and miR-191-5p among unexposed controls ($\rho_{Spearman}$=0.66) and DEE-exposed workers ($\rho_{Spearman}$=0.62).

In addition to the circulating biomarkers, the PC1 of the nasal turbinate epithelial gene expression signature was notably increased in DEE-exposed versus unexposed subjects at EC concentrations <106 µg/m^3 and <50 µg/m^3 (figure 1, table 2). There was some evidence that this gene expression signature was also altered at EC <20 µg/m^3; however, the FDR was not noteworthy (p=0.03, FDR=0.26). Additionally, there were noteworthy exposure-response relationships at EC concentrations below each of these exposure limits. We did not find evidence of correlation between the nasal turbinate gene expression signature and any of the *circulating serum* miRNAs (online supplemental table 2), whose general biological role is post-transcriptional regulation. However, this observation does not preclude the possibility that other miRNAs expressed *within nasal epithelial cells* could potentially influence gene expression in the same tissue.

Similar to our previous age-adjusted analyses,[13] we found that DEE-exposed workers had elevated urinary mutagenicity adjusting for age and smoking history, under the MSHA OEL (p=0.01, FDR=0.02, p_{trend}=9E-04, FDR=5E-03 for trend). Even below the more stringent EU OEL for EC, we found some evidence for a noteworthy EC exposure-response relationship for urinary mutagenicity (p_{trend}=0.01, FDR=0.05 for trend). However, we did not detect associations between DEE and urinary mutagenicity below the ACGIH recommended limit, which was likely due to sparse data.

DISCUSSION

We conducted new analyses on circulating biomarkers, urinary mutagenicity and a nasal epithelial cell gene expression signature that were previously found to be associated with DEE.[2 3 5 10 12 13 19] The new analyses were performed at EC concentrations below the MSHA OEL of 106 µg/m^3, the new EU OEL of 50 µg/m^3 and the ACGIH recommended workplace exposure limit of 20 µg/m^3. Below the MSHA OEL, we found that 15 of the circulating biomarkers were altered by DEE-exposure status. Below the EU OEL, we found that engine factory workers who were exposed long term to DEE had elevated lymphocytes, CD4+ counts, CD8+ counts and miR-92a-3p, as well as decreased CRP, MIP-1β, miR-423-3p and miR-122-5p. Interestingly, even below the

Table 2 Associations between DEE, circulating biomarker concentrations, urinary mutagenicity and a nasal turbinate gene expression signature at elemental carbon concentrations below the US Mine Safety and Health Administration OEL (106 µg/m^3), the European Union OEL (50 µg/m^3) and the American Conference of Governmental Industrial Hygienists recommendation (20 µg/m^3)

Biomarker	exp/un	β, cont	95% CI low	95% CI up	p_{trend}, cont	FDR, cont	P value, exp versus un	FDR, exp versus un
EC<106 µg/m^3								
Lymphocytes, cells/µL	53/55	3E-03	1E-03	4E-03	2E-03	5E-03	1E-03	7E-03
CD4+ T cells, cells/µL	53/55	4E-03	2E-03	6E-03	4E-04	4E-03	4E-04	4E-03
CD8+ T cells, cells/µL	53/55	2E-03	−3E-04	4E-03	0.09	0.10	8E-03	0.02
B cells, cells/µL	53/55	4E-03	5E-04	0.01	0.02	0.04	0.02	0.04
CRP, pg/mL†	53/55	−0.01	−0.02	0.00	0.05	0.06	0.02	0.03
MIP-1D, pg/mL†	53/55	5E-03	2E-03	0.01	2E-03	5E-03	9E-03	0.02
CXCL11/ITAC, pg/mL†	53/55	−3E-03	−0.01	0.00	0.05	0.06	0.05	0.05
IL-16, pg/mL†	53/55	3E-03	−7E-04	0.01	0.11	0.12	0.02	0.03
sILRII, pg/mL†	53/55	2E-03	5E-04	4E-03	0.01	0.02	0.12	0.12
SGP130, pg/mL†	53/55	2E-03	3E-04	3E-03	0.02	0.04	0.09	0.09
MIP-1β, pg/mL†	40/46	−0.01	−0.02	−4E-03	1E-03	5E-03	3E-03	0.01
Alu retroelement copy number, Alu/Alb ratio‡	52/55	6E-04	2E-04	1E-03	7E-03	2E-02	3E-02	0.04
miR-191-5p§	45/46	−0.01	−0.01	−2E-03	2E-03	5E-03	3E-03	0.01
miR-93-5p§	45/46	−2E-03	−4E-03	−8E-04	3E-03	0.01	0.03	0.04
miR-423-3p§	45/46	−0.01	−0.01	−3E-07	0.05	0.06	0.02	0.03
miR-122-5p§	45/46	−0.01	−0.01	1E-03	0.11	0.12	0.01	0.02
miR-92a-3p§	45/46	7E-04	−4E-04	2E-03	0.22	0.22	0.04	0.05
Nasal turbinate RNA expression PC1 diesel signature¶	41/38	2E-03	1E-03	3E-03	6E-07	2E-05	4E-09	8E-08
Urinary mutagenicity**	20/15	2E-01	8E-02	3E-01	9E-04	5E-03	0.01	0.02
EC<50 µg/m^3								
Lymphocytes, cells/µL	28/55	4E-03	7E-04	0.01	0.02	0.06	9E-03	0.04
CD4+ T cells, cells/µL	28/55	3E-03	−6E-04	0.01	0.09	0.13	0.02	0.05
CD8+ T cells, cells/µL	28/55	0.01	1E-03	0.01	0.01	0.05	5E-03	0.03
B cells, cells/µL	28/55	5E-03	−1E-03	0.01	0.10	0.13	0.08	0.12
CRP, pg/mL†	28/55	−0.02	−0.03	−3E-03	0.02	0.06	0.02	0.05
MIP-1D, pg/mL†	28/55	0.01	−1E-04	0.01	0.06	0.11	0.15	0.19
CXCL11/ITAC, pg/mL†	28/55	−0.01	−0.01	3E-04	0.06	0.11	0.09	0.12
IL-16, pg/mL†	28/55	0.01	−1E-03	0.01	0.12	0.13	0.07	0.11
sILRII, pg/mL†	28/55	2E-03	−1E-03	0.01	0.21	0.22	0.68	0.68
SGP130, pg/mL†	28/55	2E-03	−1E-03	4E-03	0.24	0.24	0.57	0.60
MIP-1β, pg/mL†	23/46	−0.01	−0.02	2E-03	0.10	0.13	0.04	0.09
Alu retroelement copy number, Alu/Alb ratio‡	27/55	7E-04	−2E-04	2E-03	0.12	0.13	0.24	0.27
miR-191-5p§	26/46	−0.01	−0.01	−5E-04	0.04	0.07	0.06	0.11
miR-93-5p§	26/46	−2E-03	−5E-03	3E-04	0.08	0.13	0.22	0.26
miR-423-3p§	26/46	−0.01	−0.02	−1E-03	0.03	0.06	0.04	0.09
miR-122-5p§	26/46	−0.02	−0.03	−5E-03	0.01	0.05	2E-03	0.02
miR-92a-3p§	26/46	2E-03	3E-04	4E-03	0.03	0.06	0.02	0.05
Nasal turbinate RNA expression PC1 diesel signature¶	23/38	3E-03	2E-03	5E-03	7E-06	2E-04	1E-06	2E-05
Urinary mutagenicity**	12/15	0.19	5E-02	3E-01	0.01	0.05	0.07	0.11
EC<20 µg/m^3								
Lymphocytes, cells/µL	6/55	0.01	−0.01	0.03	0.19	0.33	0.07	0.26
CD4+ T cells, cells/µL	6/55	0.01	−0.01	0.03	0.19	0.33	0.07	0.26
CD8+ T cells, cells/µL	6/55	0.01	−0.02	0.03	0.60	0.71	0.18	0.33
B cells, cells/µL	6/55	0.02	−0.01	0.05	0.19	0.33	0.13	0.30
CRP, pg/mL†	6/55	−0.05	−0.12	0.02	0.15	0.33	0.20	0.34
MIP-1D, pg/mL†	6/55	−3E-03	−0.03	0.03	0.85	0.90	0.50	0.59
CXCL11/ITAC, pg/mL†	6/55	−0.01	−0.04	0.03	0.72	0.80	0.82	0.82
IL-16, pg/mL†	6/55	0.03	1E-03	0.07	0.04	0.24	0.05	0.26
sILRII, pg/mL†	6/55	−0.02	−0.03	2E-03	0.08	0.30	0.10	0.27
SGP130, pg/mL†	6/55	−0.01	−0.02	0.01	0.30	0.39	0.35	0.45
MIP-1β, pg/mL†	6/46	−0.02	−0.06	0.02	0.31	0.39	0.27	0.43
Alu retroelement copy number, Alu/Alb ratio‡	6/55	−2E-03	−0.01	2E-03	0.31	0.39	0.15	0.32
miR-191-5p§	6/46	−0.03	−0.06	7E-05	0.05	0.24	0.31	0.45

continued

Table 2 continued

Biomarker	exp/un	β, cont	95% CI low	95% CI up	p_{trend}, cont	FDR, cont	P value, exp versus un	FDR, exp versus un
miR-93-5p§	6/46	−0.01	−0.02	0.01	0.30	0.39	0.68	0.72
miR-423-3p§	6/46	−0.06	−0.10	−0.01	0.01	0.19	0.06	0.26
miR-122-5p§	6/46	−0.04	−0.10	0.01	0.14	0.33	0.10	0.27
miR-92a-3p§	6/46	0.01	−2E-03	0.01	0.16	0.33	0.36	0.45
Nasal turbinate RNA expression PC1 diesel signature¶	5/38	0.01	1E-03	0.02	0.02	0.19	0.03	0.26
Urinary mutagenicity**	5/15	0.02	−0.36	4E-01	0.91	0.91	0.65	0.72

Multivariable linear regression models were adjusted for age and smoking status (never, former, current). Biomarkers were natural log transformed. We selected high prior probability biomarkers with p<0.05 (DEE-exposed vs unexposed) or p_{trend}<0.05 for EC from the source publications. Biomarkers with both p<0.05 and FDR<0.20 for DEE-exposed versus unexposed controls at EC<106 µg/m^3, and at either EC<50 µg/m^3 or EC<20 µg/m^3 were considered noteworthy.
*Lan *et al*.[3]
†Bassig *et al*.[2]
‡Dai *et al*.[19]
§Hu *et al*.[12]
¶Drizik *et al*.[10]
**Wong *et al*.[13]
Alb, albumin; Alu, arthrobacter luteus; CD, cluster of differentiation; cont, continuous; CRP, C reactive protein; CXCL, chemokine (C-X-C motif) ligand; DEE, diesel engine exhaust; EC, elemental carbon; exp, DEE-exposed workers; FDR, false discovery rate; IL, interleukin; ITAC, interferon-inducible T cell alpha chemoattractant; MIP, macrophage inflammatory protein; miR, microRNA; OEL, occupational exposure limit; PC, principal component; sILR, soluble interleukin receptor; un, unexposed controls.

ACGIH recommendation, which has DEE levels comparable to highly polluted cities and urban areas, we found some indication of EC exposure-response relationships for IL-16 and miR-423-3p. Additionally, we found that the diesel gene expression signature in chronically and directly exposed nasal epithelial tissue was altered at EC concentrations below the MSHA and EU OELs. We also found evidence of positive associations between DEE exposure and urinary mutagenicity under the MSHA and EU OELs even after accounting for age and smoking history. Our findings suggest that even at low DEE concentrations under existing, current or recommended OELs, DEE may induce early biological changes that could reflect the key characteristics of carcinogens,[11] including inflammatory/immune function and genotoxicity. Therefore, more stringent OELs may be warranted to protect the health of DEE-exposed workers.

Our most interesting and noteworthy finding was for the non-coding miRNA, miR-423-3p, a cell cycle regulator that has been linked to lung cancer progression.[21–24] In a study of non-small cell lung cancer (NSCLC) specimens and adjacent noncancerous lung tissues, miR-423-3p was previously found to be downregulated by ZNF674-AS1, which suppressed NSCLC growth.[24] In addition to miR-423-3p, miR-92a-3p was also found to be noteworthy. MiR-92a-3p was previously found to have 3.42-fold greater expression in plasma of patients with lung adenocarcinoma compared with healthy controls[25]; however, its biological mechanism is unclear.

Although the carcinogenic mechanism underlying miR-423-3p is under investigation, studies have suggested that miR-423-3p directly targets p21,[26 27] a notable gene product that has been shown to act dually as a cell cycle inhibitor and antiproliferative effector,[26 27] as well as an anti-apoptotic agent and oncogenic factor in a p53-deficient environment.[28] The dual nature of p21 potentially explains the association between increased carcinogenic DEE and decreased miR-423-3p. The mechanism by which DEE affects circulating miRNA concentrations, particularly miR-423-3p, is unclear and potentially involves both direct genotoxicity to circulating miRNAs, as well as promotion of localised tissue cytotoxicity leading to leakage of tissue-specific miRNAs into circulating blood.[12] A randomised cross-over study found that levels of several miRNAs (ie, miR-21, miR-30e, miR-215 and miR-144) were altered in response to controlled diesel exposure, and the effects were attenuated with supplementation with the antioxidant N-acetylcysteine.[29] These findings suggest that oxidative stress, which is involved in genotoxicity and cytotoxicity, may play a key role in biological alterations to circulating miRNA levels in response to carcinogenic environmental exposures.

The trend estimates for some of the noteworthy biomarkers, including miR-423-3p and nasal turbinate gene expression, increased in magnitude with decreasing OELs (ie, <106 µg/m^3 to <20 µg/m^3). At face value, these observations suggest potential non-linear exposure-response relationships across the range of EC exposure, with more pronounced effects of DEE occurring at lower EC exposure levels. However, we cannot discount the possibility that these observations are due to imprecise estimates from limited data for DEE-exposed subjects at the lower exposure thresholds (ie, EC<20 µg/m^3).

We found that lymphocyte cell counts, including CD4+ and CD8+, were elevated among DEE-exposed subjects at EC concentrations <50 µg/m^3 and <106 µg/m^3. Prospective studies have consistently found associations between increased overall white blood cell counts, which reflect subclinical inflammatory response, and increased risk of lung cancer,[30–32] even among never-smokers.[33] Similar to our previous analyses across the full range of EC exposure,[2] we found that levels of CRP, a non-specific acute-phase protein, were decreased with increasing EC at concentrations <50 µg/m^3 and <106 µg/m^3. This inverse DEE-CRP association was detected in our cross-sectional study at a single time point and the dynamics of this relation over longer periods of time are unclear. Our study was conducted in predominantly never-smokers and light-smokers and our observed inverse DEE-CRP association is consistent with some previous evidence linking lower CRP levels to increased lung cancer risk among never-smokers.[8 34] Previous prospective cohort studies have suggested effect modification of CRP-lung cancer associations by smoking status, histological subtype and potentially sex.[8 34–38] The seminal meta-analysis in the Lung Cancer Cohort Consortium found positive associations between CRP and lung cancer risk among former smokers and current smokers, and inverse associations among never-smokers.[34] Additionally, they found marginally non-significant inverse associations between CRP and lung adenocarcinoma, the subtype more common among never-smokers as well as positive associations between CRP and squamous cell carcinoma,

which is more common among smokers. We also found inverse associations between CRP and lung cancer, mainly for lung adenocarcinoma, among never-smoking Asian women.[8] Similarly, a study in the UK Biobank found positive associations between CRP and lung cancer risk among former smokers and current smokers, but no significant association among never-smokers.[35] The observed positive CRP-lung cancer association among men in meta-analyses by Muller *et al* may have been attributed to a higher proportion of smokers and squamous cell carcinoma among men, compared with women.[34] Future prospective studies of larger sample size are needed to investigate the utility of CRP as a biomarker of lung cancer risk in subgroups defined by smoking, histological subtype, sex and race/ethnicity.

Our study had notable strengths. First, we conducted detailed personal air monitoring among the DEE-exposed engine factory workers, which provided rich information on EC exposure dynamics across the work shift. Second, we collected a variety of biospecimens including blood, urine and nasal epithelial cells directly after exposure assessment, which allowed the evaluation of both early systemic and localised biological effects of DEE that may be relevant to the mechanisms of carcinogenesis.[11] Third, the wide difference in DEE concentrations among the exposed workers and unexposed controls improved the ability to detect associations. Even at the lower range of EC concentrations, the DEE levels in our study were comparable to some polluted urban environments,[6] which improves the external validity of our findings to men in the general population.

Our study had some limitations. This was a cross-sectional analysis, which limits the ability to evaluate temporality and long-term trends between exposures and biomarkers. However, it is highly unlikely that the biomarkers reflect biological processes that would affect the subjects' occupational exposure to diesel exhaust across time (ie, reverse causation). We only had several DEE-exposed subjects at EC concentrations $<20\,\mu g/m^3$, which limited statistical power for the analyses below the ACGIH recommended limit. However, even with sparse data at $EC<20\,\mu g/m^3$, we found that miR-423-3p and the nasal epithelium gene expression signature had consistent DEE associations as $EC<50\,\mu g/m^3$ and $<106\,\mu g/m^3$, which provided greater confidence in our findings. Our study was restricted to men because of the sex/gender composition of the diesel engine factory workers. Although the generalisability of our findings is limited to men, confounding by sex/gender was eliminated in our study. Lastly, the biological role of some of our main findings (ie, miR-92a-3p, miR-191-5p and miR-423-3p) in relation to lung carcinogenesis has yet to be extensively investigated and would require further characterisation.

In summary, we found that long-term occupational exposure to DEE was associated with alterations to some circulating biomarkers, a nasal epithelium gene expression profile and potentially urinary mutagenicity, even at EC concentrations below the new occupational exposure limit adopted by the EU ($50\ \mu g/m^3$). Furthermore, we found some evidence that one circulating miRNA (miR-423-3p) and a nasal epithelium gene expression profile were associated with DEE exposure, even below the ACGIH recommended exposure limit for EC ($20\ \mu g/m^3$). Our findings suggest that even low levels of exposure to DEE may result in early biological changes to markers that have been or are potentially linked to lung cancer pathogenesis. Therefore, more stringent occupational exposure limits, such as those adopted by the Netherlands ($10\ \mu g/m^3$), may be warranted to protect the respiratory health of DEE-exposed workers.

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