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ORIGINAL ARTICLE

# Bulky DNA adducts, microRNA profiles, and lipid biomarkers in Norwegian tunnel finishing workers occupationally exposed to diesel exhaust

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## ABSTRACT

**Objectives** This study aimed to assess the biological impact of occupational exposure to diesel exhaust (DE) including DE particles (DEP) from heavy-duty diesel-powered equipment in Norwegian tunnel finishing workers (TFW).

**Methods** TFW (n=69) and referents (n=69) were investigated for bulky DNA adducts (by <sup>32</sup>P-postlabelling) and expression of microRNAs (miRNAs) (by small RNA sequencing) in peripheral blood mononuclear cells (PBMC), as well as circulating free arachidonic acid (AA) and eicosanoid profiles in plasma (by liquid chromatography–tandem mass spectrometry).

**Results** PBMC from TFW showed significantly higher levels of DNA adducts compared with referents. Levels of DNA adducts were also related to smoking habits. Seventeen miRNAs were significantly deregulated in TFW. Several of these miRNAs are related to carcinogenesis, apoptosis and antioxidant effects. Analysis of putative miRNA-gene targets revealed deregulation of pathways associated with cancer, alterations in lipid molecules, steroid biosynthesis and cell cycle. Plasma profiles showed higher levels of free AA and 15-hydroxyeicosatetraenoic acid, and lower levels of prostaglandin D<sub>2</sub> and 9-hydroxyoctadecadienoic acid in TFW compared with referents.

**Conclusion** Occupational exposure to DE/DEP is associated with biological alterations in TFW potentially affecting lung homeostasis, carcinogenesis, inflammation status and the cardiovascular system. Of particular importance is the finding that tunnel finishing work is associated with an increased level of DNA adducts formation in PBMC.

## INTRODUCTION

Occupational exposure to diesel exhaust (DE) is a great health concern,<sup>1,2</sup> and is associated with respiratory and cardiovascular diseases (CVD).<sup>3</sup> DE is classified as carcinogenic to humans,<sup>2</sup> and this is supported by in vitro studies.<sup>4,5</sup> Of particular concern is the use of heavy-duty diesel engines in enclosed environments such as underground mining and tunnel construction.<sup>1</sup> Tunnel finishing workers (TFW) are fitters performing work related to, that is, electrical installation, rock support, and water and frost protection in road and railway tunnels. Despite reduction efforts, TFW are highly exposed

## Key messages

### What is already known about this subject?

- Exposure to diesel exhaust (DE) including DE particles (DEP) is carcinogenic to humans, and is associated with respiratory and cardiovascular diseases.
- Despite changes in diesel technology and stringent emission restrictions, high air concentrations of DE/DEP from heavy-duty diesel-powered equipment during tunnel finishing work in Norway are reported.
- Biological effects related to occupational exposure to DE in tunnel finishing workers are not known.

### What are the new findings?

- Occupational exposure to DE following tunnel finishing work in Norway is associated with increased levels of bulky DNA adducts and deregulation of microRNAs in peripheral blood mononuclear cells, as well as altered plasma arachidonic acid and eicosanoid profiles.

### How might this impact on policy or clinical practice in the foreseeable future?

- More attention should be directed towards clarifying biological effects of occupational exposure to DE, in particular in relation to tunnel finishing work.
- This study may have implications for risk assessment and preventive strategies concerning occupational exposure to DE.

to DE from heavy-duty diesel-powered equipment.<sup>6</sup> Using elemental carbon (EC) as a marker of DE, the geometric mean (GM) EC concentration measured from personal full-shift air samplers obtained from three tunnels during finishing work was 37.8 µg/m<sup>3</sup> (SD=2.7 µg/m<sup>3</sup>). GM level of NO<sub>2</sub> was 128.0 µg/m<sup>3</sup> (SD=2.9 µg/m<sup>3</sup>).<sup>6</sup> The biological impact of working in such an environment is not clear.

Several of the observed adverse effects of DE are associated with the particulate phase.<sup>3</sup> DE particles (DEP) are composed of EC cores with various amounts of adsorbed compounds, including polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs and some metals.<sup>3</sup> The chemical characteristics of DEP



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are affected by the fuel source, engine-type, exhaust after-treatment techniques, and operating conditions.<sup>1</sup> Exposure to DEP increases airway and systemic inflammation, which may induce oxidative stress and DNA damage.<sup>3,5,7</sup> Gaseous NO<sub>2</sub> in DE may also cause proinflammatory reactions and oxidative stress,<sup>8</sup> and an independent role for both acute and chronic NO<sub>2</sub> exposure as risk factors for CVD development has been indicated.<sup>9</sup>

PAHs induce cytochrome P450 monooxygenases, which are key metabolising enzymes in PAH-bioactivation to diol-epoxides, radical cations and o-quinones.<sup>10</sup> These reactive metabolites can directly affect DNA by forming adducts and initiating carcinogenesis. Peripheral blood mononuclear cells (PBMC) are easily accessible and have been identified as potential surrogates for PAH-exposed lung tissue.<sup>11</sup> An association between DNA adduct levels in white blood cells (WBC) and risk of lung cancer in highly PAH-exposed individuals has been reported.<sup>12</sup> Significantly increased levels of DNA adducts were found in lymphocytes from DE-exposed bus maintenance and truck terminal workers,<sup>13,14</sup> and a recent study reported significantly increased levels of DNA-strand breaks in leucocytes following DE exposure.<sup>15</sup>

Epigenetic changes are strongly associated with environmental exposures and are mechanistically linked to adverse health effects.<sup>16</sup> miRNA are single-stranded, short non-coding RNA sequences (~22 nucleotides) that post-transcriptionally can regulate gene expression. Deregulations of several miRNAs were identified from studies on exposure to DEP and air pollution.<sup>16</sup> Altered expression of four miRNAs in PBMC after controlled short-term exposure of human participants to DE has been reported.<sup>17</sup> In a study of foundry workers exposed to metal-rich particulate matter (PM), several miRNAs affecting candidate inflammatory genes were found to be deregulated.<sup>18</sup> Little is known, however, about effects of occupational exposure to DE/DEP on miRNA expression, in particular regarding tunnel finishing work.

Enzymatic and non-enzymatic oxidation of arachidonic acid (AA) and its precursor linoleic acid yields prostaglandins (PGs), hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acid (HODE) lipid mediators with implications for inflammation, CVD and carcinogenesis.<sup>7,19,20</sup> Plasma lipid peroxidation products are suitable biomarkers of the biologically effective dose of PM and are shown to reflect oxidative stress in the airways.<sup>21</sup> Increased levels of 8-iso-PGF<sub>2α</sub>, 12-HETE and 13-HODE were found in plasma from DE-exposed mice.<sup>7</sup> A role for lipid peroxidation in mediating PM-induced atherosclerosis was reported in mice with increased plasma levels of 9-HODE and 12-HETE.<sup>22</sup> Additionally, proinflammatory and cancer-promoting effects were reported for PGE<sub>2</sub> and by contrast, PGD<sub>2</sub> was identified as suppressor of cell invasion and generally was reported as antitumorigenic.<sup>19</sup> Increased levels of 5-HETE, 12-HETE and 15-HETE are also linked to carcinogenesis and CVD.<sup>7,20</sup> To our knowledge, however, effects on circulating AA and eicosanoids in human plasma following occupational exposure to DE/DEP have not been investigated.

This study investigated the biological impact of DE/DEP exposure associated with tunnel finishing work. We aimed to identify effect biomarkers in PBMC and plasma from TFW compared with a referent group. Bulky DNA adducts and miRNA expression profiles in PBMC were measured by <sup>32</sup>P-postlabelling and small RNA sequencing (sRNA-seq), respectively. Circulating free plasma AA and eicosanoids were analysed by liquid chromatography–tandem mass spectrometry (LC/MS/MS). Finally, expression of selected protein coding genes related to PAH-bioactivation and DNA adduct formation was explored by RT-qPCR.

## MATERIALS AND METHODS

### Study group

An occupational cross-sectional study was performed in Norwegian male TFW (n=69) and referents (n=69) working at the same/similar construction sites. In addition to a high level of exposure to DEP, some exposure to concrete dust may occur in TFW. Referents are administrative personnel, engineers, and outdoor road workers. Participants have mainly similar socioeconomic status. Referents may occasionally work inside tunnels for shorter time periods, and some exposure to asphalt and vehicle emission may occur. Generally, participants work for 11–12 days consecutively with 10–12 hours shifts, before a 9-day period off work. At the time of enrolment (2015–2016), the participants were employed by five different Norwegian companies working at eight construction sites in South Norway.

Blood samples were collected from each participant on a single occasion at the construction sites in between shifts and each participant filled out a questionnaire. Background information collected included work operations, age, body mass index (BMI), smoking habits: never-smoker; former-smoker (less than a year); former-smoker (more than a year); daily-smoker; party-smoker, use of respirators (yes, often; yes, rarely; no), respiratory diseases (chronic bronchitis, chronic obstructive lung disease) and allergies.

### Sample collection

Blood was collected into EDTA tubes (BD Vacutainer K2E, Mediq Norge AS, Kløfta, Norway) and kept rotating EDTA blood was loaded onto a SepMate tube (StemCell Technologies, Cambridge, UK) preloaded with Ficoll (GE Healthcare, Oslo, Norway). Plasma was separated according to the manufacturer's instructions and stored at –80°C. PBMC were treated with RBC lysis buffer and stored as pellets at –80°C.

### <sup>32</sup>P-postlabelling

DNA was isolated from PBMC by standard phenol/chloroform extraction. The nuclease P<sub>1</sub> enrichment version of the thin-layer chromatography <sup>32</sup>P-postlabelling assay was used to determine bulky DNA adduct formation. The procedure was performed essentially as described,<sup>10</sup> with minor modifications. All samples were analysed blind and in duplicate. An external benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)-modified DNA-standard was used as a positive control. Total DNA adduct levels were considered representative of PAH-DNA and other aromatic/hydrophobic adducts resistant to nuclease P<sub>1</sub> digestion and measured in the diagonal radioactive zone. The method provides a summary measure of a complex mixture of adducts present in the postlabelling chromatograms. Results are expressed as adducts/10<sup>8</sup> nucleotides. Representative autoradiographic profiles of DNA adducts from never-smoking referents and TFW, daily-smoking TFW and an external BPDE-modified DNA-standard positive control are shown in online supplementary figure 1.

### Isolation of total-RNA

RNA was isolated from PBMC using Trizol (Thermo Fisher Scientific, Oslo, Norway), dissolved in nuclease free water, and stored at –80°C. RNA-integrity number (RIN) was determined on an Agilent Bioanalyzer with RNA 6000 Nano kit (Agilent Technologies, Oslo, Norway). RIN values were in all cases ≥7.0.

## Measurement of gene expression

RNA was reverse transcribed with the qScript complimentary DNA (cDNA) synthesis kit (Quanta Biosciences, VWR International, Oslo, Norway). qPCR reactions were set up with PerfeCTa SYBR Green Fastmix (ROX) (Quanta Biosciences, VWR International). Relative gene expression was normalised to the expression of RNA18S and calculated using the  $\Delta\Delta C_t$  method. Primers (online supplementary table 1) were purchased from Sigma-Aldrich or Thermo Fisher Scientific.

## Small RNA sequencing (sRNA-seq)

Libraries for sRNA-seq were prepared using QIAseq miRNA Library kit (Qiagen, Hilden, Germany). Briefly, adapters were ligated to the 3' and 5' ends of miRNAs. sRNAs with ligated adapters underwent reverse transcription with unique molecular identifier (UMI) assignment followed by library amplification (16 cycles) on a Mastercycler Nexus (Eppendorf, Hamburg, Germany). sRNA libraries were validated on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California, USA) using high-sensitivity DNA chips. Sequencing of sRNA libraries was performed on MiSeq system (Illumina, San Diego, California, USA) using MiSeq Reagent Kit V.3.

## Analysis of AA and eicosanoids

Solid-phase extraction was used for AA metabolites (AA, PG, HETE, HODE, epoxyeicosatrienoic acids and leukotriene  $A_4$ ) from 0.5 mL human plasma samples. Analysis of plasma AA and eicosanoids by LC/MS/MS was performed as previously described.<sup>4</sup> PGI<sub>2</sub> was determined as its non-enzymatically hydrolysed product 6-keto-PGF<sub>1 $\alpha$</sub> .

## Statistics

Data were analysed in StataSE V.14. DNA adduct measurements and gene expression data were log-transformed to normalise the distribution of the error terms and analysed by linear regression. Differential miRNA expression count data were generated by QIAseq miRNA Quantification, and the UMI counts were taken to compensate for sequencing bias. The DESeq2-package was used to test for differential expression by the application of negative binomial generalised linear models. Differential expression in experimental designs with two levels of a factor (TFW vs referents) was tested by Wald. A  $p < 0.02$  was used as cut-off for statistically significant deregulated miRNAs between TFW and referents. miRNA target genes and associated pathways were identified using DIANA mirPath V.3 and TarBase V.7.0 (accessed July 2018) with  $p < 0.05$  (Fisher's exact test, false discovery rate (FDR) correction). Prior to analysis, some of the eicosanoid variables were transformed, using a square-root, log, or inverse transformation, to obtain a closer fit to the normal distribution for the residuals. Each variable was analysed by linear regression. Smoking was included in the analyses, while age and BMI were only added if  $p < 0.1$ . Based on likelihood ratio tests, the residual variance was allowed to differ between referents and TFW for one outcome variable.

## RESULTS

### Subject characteristics

TFW (n=69) and referents (n=69) participated in the study by giving one blood sample during a random workday in addition to filling out a questionnaire. All subjects were Caucasian men. Few workers reported wearing protective respirators or filtering half masks. Compared with referents, TFW were significantly younger and were more inclined to tobacco smoking (table 1). Other

**Table 1** Demographics

	Referents	TFW	P values
Participants	n=69 (%)	n=69 (%)	
Age			
Overall mean $\pm$ SD	50.0 $\pm$ 13.2	41.0 $\pm$ 12.2	6.0 $\times$ 10 <sup>-5*</sup>
Gender (%)			
Male	69 (100)	69 (100)	
Ethnic group (%)			
Caucasian	69 (100)	69 (100)	
Smoking history (%) <sup>‡</sup>			0.003 <sup>†</sup>
Never-smoker	28 (41)	17 (25)	
Former-smoker (less than a year)	1 (2)	6 (9)	
Former-smoker (more than a year)	19 (28)	11 (16)	
Daily-smoker	8 (12)	24 (35)	
Party-smoker	11 (16)	11 (16)	
BMI (SD) <sup>§</sup>	27.5 (3.7)	27.2 (3.9)	
Respirator mask (%) <sup>¶</sup>			0.001 <sup>†</sup>
Yes, often	1 (1)	5 (7)	
Yes, rarely	26 (38)	43 (62)	
No	42 (61)	20 (29)	
Respiratory diseases (%) <sup>**††</sup>			
Chronic bronchitis	3 (4)	1 (1)	
Chronic obstructive lung disease	1 (1)	0 (0)	

\*Student's t-test.

<sup>†</sup>Pearson  $\chi^2$ .

<sup>‡</sup>Two cases (4%) from the referent group were missing data on smoking status.

<sup>§</sup>Eight cases (12%) from the referent group and two cases (3%) from the TFW group were missing data on BMI.

<sup>¶</sup>One case (1%) from the TFW group was missing data on use of respirators.

\*\*One case (1%) from the referent group and four cases (6%) from the TFW group were missing data on chronic bronchitis.

<sup>††</sup>Five cases (7%) from the referent group and two cases from the TFW group were missing data on chronic obstructive lung disease.

BMI, body mass index; TFW, tunnel finishing worker.

relevant parameters were not significantly different between TFW and referents.

### Bulky DNA adducts

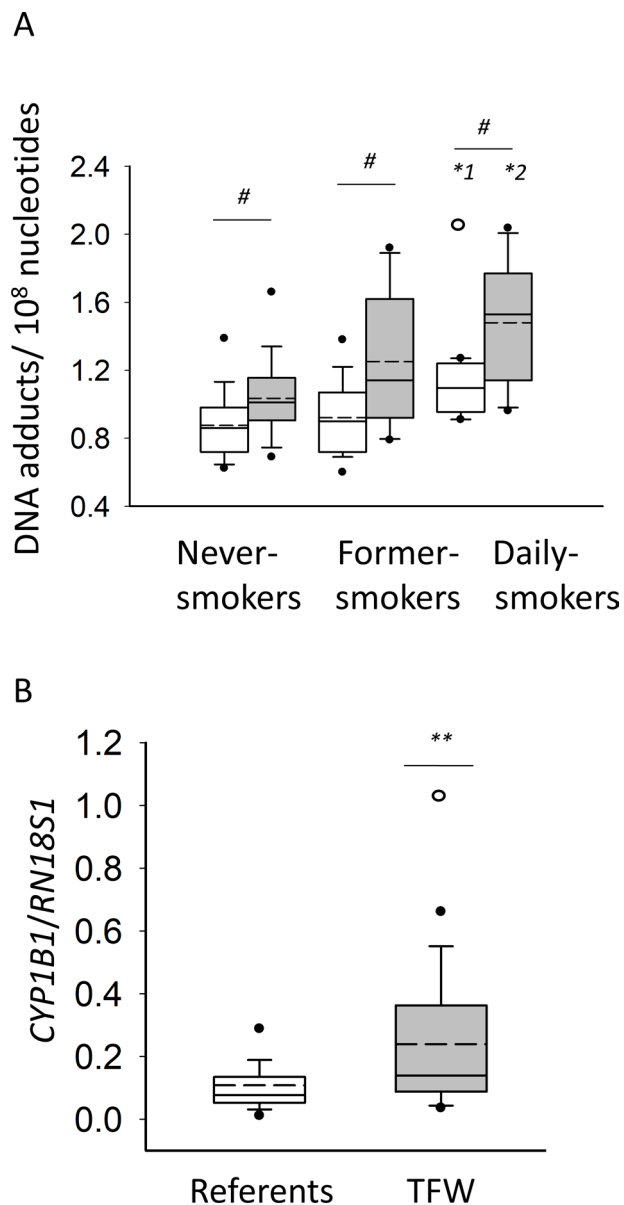
When stratified by smoking, significantly more DNA adducts were measured in PBMC from TFW compared with referents. In the referent group, daily-smokers had significantly more DNA adducts compared with never-smokers. Equivalently, daily-smoking TFW had significantly more DNA adducts compared with never-smoking TFW (figure 1A).

### Gene expression of CYP1A1 and CYP1B1

Expression of CYP1A1 and CYP1B1 was measured in PBMC from never-smoking and daily-smoking TFW and referents. Significantly higher expression of CYP1B1 was found among TFW compared with referents (figure 1B). Interestingly, there were no effect modifications by smoking. Expression of CYP1A1 was below the detection limit, and expressions of AHR and ARNT were not significantly different between the groups (data not shown).

### Identification of deregulated miRNAs and associated pathways

sRNA-seq was performed on samples from never-smoking TFW (n=14) and referents (n=13) matched by age and BMI.



**Figure 1** Bulky DNA adducts and CYP1B1 gene expression in PBMC from TFW and referents. (A) DNA adducts per 10<sup>8</sup> nucleotides in three smoking categories; never-smokers (n=27/17), former-smokers (n=19/16) and daily-smokers (n=7/21). (B) Gene expression of CYP1B1 in referents (n=36) and TFW (n=41). Box plots show 5th/95th percentile, median (solid line) and mean (dotted line). Referents are shown in white and TFW in grey boxes. Two extreme outliers marked with unfilled circles (one referent, daily-smoker (A) and one TFW, never-smoker (B) are excluded from the statistical analysis. P<0.05 (linear regression). \*, significance between never-smokers and daily-smokers within the referents (\*<sup>1</sup>) and the TFW group (\*<sup>2</sup>), respectively. \*\*, significance between referents and TFW. #, significance between referents and TFW within each smoking category. TFW, tunnel finishing worker.

Seventeen miRNA (13(↓), 4(↑)) were identified as deregulated in TFW compared with referents (p<0.02) (table 2).

Experimentally supported miRNA-gene interactions followed by pathway analysis were obtained for deregulated miRNAs (table 2) by DIANA mirPath and TarBase. Ten Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were significantly deregulated based on presumptive miRNA-gene target interactions (table 3). The pathways fatty acid

**Table 2** Deregulated miRNAs identified by sRNA-seq

miRNA	log2FoldChange	P values
hsa-miR-4700-5p	-0.80	0.002
hsa-miR-5701	-0.75	0.002
hsa-miR-340-3p	-0.48	0.003
hsa-miR-6513-3p	0.44	0.009
hsa-miR-20b-5p	0.47	0.010
hsa-miR-3614-5p	-0.63	0.010
hsa-miR-106a-3p	0.57	0.011
hsa-miR-660-3p	-0.59	0.012
hsa-miR-4288	-0.56	0.012
hsa-miR-31-5p	0.45	0.013
hsa-miR-23a-5p	-0.57	0.013
hsa-miR-196b-5p	-0.54	0.015
hsa-miR-3130-3p	-0.61	0.015
hsa-miR-6808-3p	-0.57	0.016
hsa-miR-548an	-0.51	0.017
hsa-miR-6828-5p	-0.60	0.018
hsa-miR-652-3p	-0.52	0.019

The table display deregulated miRNAs p<0.02 (Wald) as identified by sRNA-seq. sRNA-seq, small RNA sequencing.

biosynthesis and metabolism, steroid biosynthesis, proteoglycans in cancer and cell cycle may be of particular importance. Further information regarding miRNA-gene targets and their clustering are found in online supplementary table 2 and figure 2, respectively.

### Circulating AA and eicosanoids

Circulating free plasma AA and eicosanoids were measured in a subset of never-smoking and daily-smoking TFW and referents (table 4). Significantly more AA and 15-HETE, and significantly less PGD<sub>2</sub> were found in TFW compared with referents. Compared with never-smoking referents, significantly less 9-HODE was measured in never-smoking TFW. Concentrations of all measured metabolites, although not significantly affected, are found in online supplementary table 3.

**Table 3** Deregulated KEGG pathways associated with miRNA-gene targets

Deregulated pathways	No miRNAs	No genes	P values
Fatty acid biosynthesis	1	1	<1.0E-325
Prion diseases	1	4	3.9E-12
Steroid biosynthesis	4	3	3.6E-07
Fatty acid metabolism	1	1	6.7E-03
Transforming growth factor-β signalling pathway	3	20	1.1E-02
Viral carcinogenesis	3	32	1.7E-02
Pantothenate and coenzyme A biosynthesis	1	1	3.5E-02
Lysine degradation	6	13	3.8E-02
Proteoglycans in cancer	4	42	4.4E-02
Cell cycle	3	27	4.8E-02

Deregulated KEGG pathways associated with genes targeted by miRNA identified from sRNA-seq (table 3) using mirPath V.3 and TarBase V.7.0. P<0.05 (Fisher's exact test, FDR correction). Further information regarding miRNAs and genes is provided in online supplementary table 2. sRNA-seq, small RNA sequencing.



**Table 4** Circulating levels of AA and eicosanoids significantly differing in referents and TFW

Metabolite*	Group	Subgroup	n	Mean (SD)	Median (Q)†	P values‡	
AA§ (invers)	Referents	Never-smokers	13	217.1 (34.5)	207.0 (164.0, 288.7)	x¶	y**
		Daily-smokers	8	249.0 (96.5)	220.4 (166.1, 480.5)	0.412	
	TFW	Never-smokers	14	259.8 (81.2)	235.8 (190.6, 504.0)	0.086	<b>0.002</b>
		Daily-smokers	24	336.6 (124.9)	298.5 (195.1, 577.3)	<b>&lt;0.001</b>	
PGD <sub>2</sub> (square root)	Referents	Never-smokers	12	24.7 (13.0)	22.9 (8.1, 48.5)	x¶	y**
		Daily-smokers	8	24.4 (14.1)	23.9 (5.0, 44.8)	0.852	
	TFW	Never-smokers	14	15.9 (6.3)	16.0 (7.5, 28.0)	<b>0.034</b>	<b>0.002</b>
		Daily-smokers	24	14.9 (8.3)	13.9 (4.0, 27.9)	<b>0.004</b>	
15-HETE (log)	Referents	Never-smokers	13	27.4 (9.9)	25.6 (17.1, 48.4)	x¶	y**
		Daily-smokers	8	36.4 (24.6)	29.9 (8.5, 73.6)	0.672	
	TFW	Never-smokers	14	36.1 (18.0)	34.2 (8.5, 79.7)	0.311	<b>0.041</b>
		Daily-smokers	23	48.9 (23.3)	44.8 (17.1, 83.4)	<b>0.005</b>	
9-HODE (log)	Referents	Never-smokers	13	349.0 (163.2)	348.9 (117.7, 636.1)	x¶	y**
		Daily-smokers	8	336.7 (273.1)	211.7 (91.1, 906.4)	0.614	
	TFW	Never-smokers	14	212.3 (162.6)	146.4 (67.3, 617.8)	<b>0.033</b>	0.063
		Daily-smokers	24	334.1 (326.5)	230.5 (60.6, 976.4)	0.22	

\*pg/mL, transformation method.

†Quantiles: 5%, 95%.

‡Linear regression. Significant p values (p&lt;0.05) in bold.

§ng/mL.

¶x (referents, never-smokers) and \*\*y (referents, never-smokers and daily-smokers combined) denote the reference groups for the corresponding statistical analyses.

AA, arachidonic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 9-HODE, 9-hydroxyoctadecadienoic acid; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>; TFW, tunnel finishing worker.

## DISCUSSION

Detailed exposure measurements of dust, EC, organic carbon, and NO<sub>2</sub> have been reported from various job groups related to drilling and blasting during tunnel construction in Norway.<sup>23</sup> However, DE/DEP exposure from heavy-duty diesel-powered equipment during tunnel finishing work was only recently investigated.<sup>6</sup> Here, molecular alterations in PBMC and blood plasma coherent with working in such an environment were investigated. Results indicated increased levels of bulky DNA adducts and changes in miRNA profiles in PBMC, as well as altered free plasma AA and eicosanoid profiles in TFW.

Stratified by smoking, significantly more DNA adducts were measured in PBMC from TFW compared with the referents. Similarly, more DNA adducts were measured in daily-smokers than never-smokers in both groups. It is established that smoking increases DNA adducts in PBMC.<sup>11</sup> Here, results indicate that exposures encountered during present-day tunnel finishing work pose a further increased risk of developing DNA adducts in these cells. These findings are in line with previous studies investigating levels of DNA adducts in PBMC/WBC following exposure to DE,<sup>13 14 24</sup> and positive correlations between cigarette smoking and DNA adducts in PBMC.<sup>11</sup> Taking into account that DNA adducts in bronchial tissue may predict lung cancer risk,<sup>25</sup> and that adducts in PBMC can be considered a distant surrogate of pulmonary exposure,<sup>11</sup> this finding may have important implications for risk assessment and preventive strategies concerning the group of TFW.

Levels of DNA adducts are dependent on the individual's xenobiotic metabolising capability. Significantly higher levels of CYP1B1 expression were measured in PBMC from TFW compared with referents. Interestingly, CYP1B1 expression was not influenced by smoking and CYP1A1 expression in PBMC was below the detection limit in agreement with a previous report.<sup>26</sup> AHR and ARNT were equally expressed in both groups. A crosstalk between the oestrogen receptor (ER) and the AHR pathway exists.<sup>27</sup> Several PAHs present in DEP are shown to directly activate ER $\alpha$ ,<sup>27</sup> indicating that CYP1B1 may

be induced by non-AHR-mediated mechanisms. Alternatively, endogenous availability of coactivators may play a role.<sup>27</sup> Nonetheless, results suggest that CYP1B1, rather than CYP1A1 may have implications for DEP-induced DNA adduct formation in PBMC.

sRNA-seq revealed significant deregulation of 17 miRNAs in TFW compared with referents. Little is known about these miRNAs in relation to DE/DEP exposure from either occupational or air pollution studies. miR-106a-3p, being upregulated in TFW, has been reported as an oncomir in humans exposed to air pollution.<sup>16</sup> miR-23a-5p was downregulated in TFW. Downregulation of miR-23a is associated with activation of apoptotic pathways, in addition to increased expression of the antioxidant superoxide dismutase [Mn], mitochondrial, which is important in mitochondrial reactive oxygen species detoxification.<sup>28</sup> miR-31-5p was upregulated in TFW and has been found to act as tumour suppressor and apoptotic inducer in both in vitro and in vivo air pollution studies.<sup>16</sup> In agreement with the present study, downregulation of miR-652-3p was also reported in human blood after short-term PM<sub>10</sub> exposure. This miRNA has a putative role in CVD.<sup>29</sup>

The remaining deregulated miRNAs have not been studied in relation to DE/DEP exposure. Some are associated with lung and colorectal cancers and as possible prognostic markers (miR-20b-5p, miR-196b-5p, miR-4500 and miR-340),<sup>30-32</sup> while others may have roles in Alzheimer's disease, chronic heart failure, and cell migration and invasion (miR-6513-3p and miR-660-3p).<sup>16 33</sup> Both miR-20b-5p and miR-196b-5p may be important regulator of apoptosis and tumorigenesis.<sup>32</sup> Whether these miRNAs are related to adverse health effects of DE/DEP exposure is not known.

Pathway union analysis based on putative miRNA-gene targets revealed potential deregulation of several fatty acid related KEGG pathways in TFW, in addition to cell cycle, steroid biosynthesis, and proteoglycans in cancer. Exposure to air pollution is significantly associated with increased risk of type 2 diabetes mellitus.<sup>34</sup> Subchronic exposure to low concentrations

of DE is shown to deregulate genes related to fatty acid biosynthesis in mice.<sup>35</sup> miRNAs may regulate cell cycle progression,<sup>36</sup> and altered expression of cell cycle-related genes has been reported after exposure to organic components from DEP.<sup>37</sup> Steroid biosynthesis is a target for endocrine disrupting chemicals, including PAHs.<sup>27</sup> Interestingly, human bronchial epithelial cells exposed to DEP *in vitro* also showed deregulation of this pathway.<sup>4</sup> Deregulation of proteoglycans in cancer may be of importance as this pathway of extracellular matrix constituents is emerging as highly affecting homeostasis and progression of various diseases, including cancer.<sup>38</sup> Combined, results indicate potential mechanisms for associations between miRNA-regulated pathways, increased levels of DNA adducts and altered plasma AA and eicosanoid profiles in TFW. Deregulated pathways may affect lung homeostasis, carcinogenesis, inflammation status, and the cardiovascular system. Further research is needed to identify specific mechanisms behind deregulated miRNAs and associated pathways following occupational exposure to DE/DEP.

Little is known about free plasma AA and associated eicosanoids in response to DE/DEP exposure *in vivo*. In line with present findings, increased levels of plasma 15-HETE were found in PM-exposed mice.<sup>39</sup> 15-HETE signalling is associated with cardiovascular pathogenesis, as well as cancer development.<sup>20</sup> While we report reduced levels of PGD<sub>2</sub> in TFW, an increase in this metabolite was measured in plasma from PM-exposed mice.<sup>39</sup> Interestingly, the great majority of studies indicate antitumorigenic activities of PGD<sub>2</sub>.<sup>19</sup> Levels of 9-HODE was lower in never-smoking TFW compared with never-smoking referents, suggesting the involvement of antioxidant responses. Increased level of this metabolite has been measured in PM-exposed mice,<sup>22</sup> but the modulation of oxidative stress and antioxidant defence processes may be time and dose dependent. Evidence indicates that gaseous NO<sub>2</sub> is an independent risk factor for CVD.<sup>9</sup> Since NO<sub>2</sub>, together with DEP and other DE constituents, can induce oxidative stress and inflammation,<sup>35,78</sup> it may be speculated that NO<sub>2</sub> participates in AA and eicosanoid deregulation. Combined, present results suggest that in particular increased concentrations of AA and 15-HETE, and decreased levels of PGD<sub>2</sub> and 9-HODE may represent biomarkers of exposure to DE/DEP. These analytes make up a complex network with the potential to modulate inflammation, cell growth responses and the cardiovascular system, but more research is needed to elucidate in detail their biological effects.

The carcinogenicity of DE/DEP to humans has been debated.<sup>40</sup> Combined, this study indicates increased bulky DNA adducts in PBMC and deregulation of cancer and fatty acid-related pathways, as well as altered free plasma AA and eicosanoid signalling molecules, associated with working in a DE/DEP-polluted environment. Although not dealing with cancer risk *per se*, the results are in favour of DE/DEP having carcinogenic potential, which is supported by *in vitro* studies.<sup>4,5</sup>

Possible confounding due to the healthy worker effect is of general concern. Here, all participants were employed at the time of enrolment. Women were excluded because of low number of potential participants. The cross-sectional study design combined with the one blood sample obtained from each participant could have led to incidental results. However, grouping TFW and referents without major stratification limits the potential influence by such bias. The referents constitute a more heterogeneous group than the TFW and they may occasionally spend short time periods within tunnels. Combined, one could speculate that differences between TFW and referents may potentially be more pronounced than the current results indicate.

## CONCLUSION

In conclusion, exposure to DE/DEP during tunnel finishing work is associated with biological alterations in TFW. This group of workers has been little studied regarding potential work-related adverse health effects. More attention should be directed towards describing biological effects and possible implications on health outcomes from occupational exposure to DE/DEP and, in particular, related to tunnel finishing work.

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