Toxicity of diesel exhaust:
Biological effects of occupational exposure and chemical *in vitro* transformation of human bronchial epithelial cells

Thesis for the degree of Philosophiae Doctor (PhD)

University of Oslo, 2018

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STAMI
STATENS ARBEIDSMILJØINSTITUTT

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo

ISBN 978-82-8377-426-9

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

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ACKNOWLEDGEMENTS

The work presented in this thesis was carried out from February 2014 to December 2018 at the Department of Chemical and Biological Work Environment at the National Institute of Occupational Health (STAMI), Oslo. Financial funding for the project was provided by STAMI. The Statoil Working Environment Fund financially supported the *in vivo* study. I am grateful to STAMI for the opportunity to work on this project for my PhD.

I sincerely thank Dr. Steen Mollerup for being my main supervisor, mentor, and toughest critic. I thank you for your valuable support over the years. I could always count on your help, assistance, and feedback, from every minor detail to actual difficulties I have encountered. You have taught me impeccable laboratory work, scientific writing, and the importance of high standards in every aspect of scientific work. I admire your knowledge in the field and your eye for detail.

I wish to thank prof. Arne Klungland, my co-supervisor at the University of Oslo. Thank you prof. Håkan Wallin for reading over, and commenting on my thesis. To prof. em. Aage Haugen, thank you for your valuable contributions to the project design. I also want to thank all the co-authors for their contributions to the articles, this work could not have been conducted without you. Thank you, Rita Bæra for your excellent laboratory work, your patience, our conversations, and for all your help.

Thank you, Dr. Yke J. Arnoldussen and Dr. Johanna Samulin-Erdem, my two lovely cheerleaders. Your laboratory skills and analytical mind-sets are inspirational. Thank you for taking me under your wings, for the discussions, feedback, ideas, and friendship. Thank you, Yke, for reading my thesis. Your comments have been highly appreciated.

To my fellow PhD students Dr. Jolien Vleeshouwers, Lise Fløvik, Kine Askim, and Dr. Audun Bersaas, it has been incredible sharing the PhD experience with you, and I am so thankful for our friendship.

Thank you, my wonderful colleagues at STAMI for all the laughter, fun, and assistance in the laboratories, the scientific and non-scientific discussions, the lunches, the numerous coffee breaks (both with and without cake), the ice creams in the sun, the occasional glass of wine, and for your support and understanding.

To all the workers and construction companies participating in the study, thank you for your contributions to the project.

To my dear family and friends, thank you for your encouragement and patience, the numerous dinners, walks, cups of tea, listening ears, pep talks, workouts, and the chess games. It has been invaluable.

Oslo, December 2018

Iselin Rynning

ABBREVIATIONS

AA	Arachidonic acid
ADCA	Adenocarcinoma
AHR	Aryl hydrocarbon receptor
B[*a*]P	Benzo[*a*]pyrene
BMI	Body mass index
CDK	Cyclin dependent kinase
COPD	Chronic obstructive lung disease
COX	Cyclooxygenase
CSC	Cigarette smoke condensate
CVD	Cardiovascular disease
CYP	Cytochrome P450 monooxygenase
DE	Diesel exhaust
DEP	Diesel exhaust particles
E2	17β-oestradiol
EC	Elemental carbon
EMT	Epithelial-to-mesenchymal transition
ERα	Oestrogen receptor alpha
GM	Geometric mean
FDR	False discovery rate
FEV1	Forced expiratory volume during the first second
FVC	Forced vital capacity
HBEC	Human bronchial epithelial cells
HETE	Hydroxyeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
IARC	International Agency for Research on Cancer
IL	Interleukin
LA	Linoleic acid
lncRNA	Long non-coding RNA
LOX	Lipoxygenase
miRNA	microRNA
mRNA	Messenger RNA
Nitro-PAH	Nitrated PAH

NO_x	Nitrogen oxide
NSCLC	Non-small cell lung cancer
OC	Organic carbon
OEL	Occupational exposure level
OH-E2	Hydroxyoestradiol
Oxy-PAH	Oxygenated PAH
PAH	Polycyclic aromatic hydrocarbons
PBMC	Peripheral blood mononuclear cells
PG	Prostaglandin
PLA_2	Phospholipase A_2
PM	Particulate matter
QRA	Quantitative risk assessments
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCC	Squamous-cell carcinoma
SCLC	Small-cell lung cancer
sRNA	small RNA
sRNA-seq	small RNA sequencing
T2	Transformed HBEC3
T2KT	Transformed HBEC2
T12KT	Transformed HBEC12
TERT	Telomerase reverse transcriptase
TFW	Tunnel finishing workers
WBC	White blood cells

LIST OF PAPERS

Paper 1

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

Iselin Rynning, Volker M. Arlt, Kristyna Vrbova, Jiri Neca, Pavel Rossner, Jr., Jiri Klema, Bente Ulvestad, Elisabeth Petersen, Øivind Skare, Aage Haugen, David H. Phillips, Miroslav Machala, Jan Topinka, and Steen Mollerup

Occup Environ Med 2018: Epub ahead of print, doi:10.1136/oemed-2018-105445

Paper 2

"*In vitro* transformation of human bronchial epithelial cells by diesel exhaust particles: Gene expression profiling and early toxic responses"

Iselin Rynning, Jiri Neca, Kristyna Vrbova, Helena Libalova, Pavel Rossner, Jr., Jørn A. Holme, Kristine B. Gützkow, Anani K. Johnny Afanou, Yke J. Arnoldussen, Eva Hruba, Øivind Skare, Aage Haugen, Jan Topinka, Miroslav Machala, and Steen Mollerup

Toxicol Sci 2018;166:51-64

Paper 3

"Gene and microRNA expression profiles of human bronchial epithelial cells transformed *in vitro* by diesel exhaust particles, cigarette smoke condensate, or benzo[*a*]pyrene"

Iselin Rynning, Kristyna Vrbova, Pavel Rossner, Jr., Helena Libalova, Jiri Klema, Audun Bersaas, Jan Topinka, Aage Haugen, and Steen Mollerup.

Manuscript

SUMMARY

The issue of urban air pollution and lung cancer risk became a concern already in the 1950s. Of particular health concern has long been occupational settings where heavy-duty diesel powered equipment is used in enclosed areas such as underground mining and tunnel construction. Exposure to diesel exhaust (DE), including DE particles (DEP), is associated with lung and cardiovascular diseases. In 2012, exposure to DE/DEP was classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer. This classification was predominantly based on evidence of increased risk of lung cancer from studies in non-metal miners and truck drivers.

Lung cancer is the leading cause of cancer worldwide and the five-year survival rate remains low. The main risk factor for lung cancer is tobacco smoking, although contributions from environmental and occupational exposures including DE/DEP is of importance.

More knowledge is needed on the toxic effects of DE/DEP and molecular alterations associated with chemically induced lung carcinogenesis to aid in establishing occupational exposure limits and the continued development of lung cancer treatments.

In this thesis, the main objective was to investigate biological effects associated with exposures to DE/DEP, both *in vivo* in Norwegian tunnel finishing workers (TFW) and *in vitro* using human bronchial epithelial cells (HBEC). In addition, a comparative *in vitro* study was performed to assess toxic effects of DEP, cigarette smoke condensate (CSC) and benzo[a]pyrene (B[*a*]P).

Despite reduction efforts, the level of DE/DEP emitted from diesel powered equipment remains relatively high during tunnel finishing work in Norway. TFW are fitters performing work related to e.g. electrical installations, rock support, and water- and frost protection. In this thesis, increased levels of bulky DNA adducts, deregulated expression of microRNA (miRNA) in peripheral blood mononuclear cells, in addition to altered profiles of plasma arachidonic acid and eicosanoids are reported in TFW compared with a reference group.

Long-term *in vitro* exposure of a HBEC line to DEP induced the ability of these cells to form colonies in soft agar assay. A stable DEP transformed cell line, T2-HBEC3, was established. The further analyses indicated the occurrence of epithelial-to-mesenchymal transition (EMT) based on morphological alterations and expression of EMT marker genes. Parental HBEC3 and T2-HBEC3 showed basal differences in gene expression profiles, and altered sensitivity to DEP

in short-term exposure experiments particularly regarding genes involved in xenobiotic and lipid metabolism, and inflammation.

Gene- and miRNA expression profiles of T2-HBEC3 were compared with those of transformed cell lines previously established from long-term exposures to CSC and B[*a*]P. Both general and specific changes in gene- and miRNA expression profiles were identified in the transformed cell lines, which were mainly associated with carcinogenesis, EMT, and the extracellular matrix. Moreover, alterations in the aryl hydrocarbon receptor pathway and inflammatory responses were identified.

In conclusion, the studies conducted for this thesis add information of toxic effects of diesel exhaust both *in vivo* and *in vitro*, in addition to contributing to an increased understanding of molecular events during chemically induced transformation of human bronchial epithelial cell lines.

1 INTRODUCTION

1.1 Exposure to air pollution and diesel exhaust

1.1.1 Particulate matter air pollution

Particulate matter (PM) air pollution is a major health concern worldwide, and transportation, power generation, industrial activities, biomass burning, and domestic heating and cooking constitute central anthropogenic sources [1]. The first report of an association between air pollution and lung cancer was published in 1954 [2]. Since then, epidemiological and experimental studies have shown associations between exposure to PM and development of respiratory and cardiovascular diseases (CVD), and allergies [1;3-6].

1.1.2 PM exposure levels

PM can be divided into respirable particulate matter <10 µm (PM_{10}) and fine particulate matter <2.5 µm ($PM_{2.5}$), which includes the fraction of ultrafine particles <0.1 µm ($PM_{0.1}$). $PM_{2.5}$ is increasingly used as an indicator air pollutant and constitutes a heterogeneous mixture of organic and inorganic compounds [1]. It was reported in several studies that $PM_{2.5}$ concentrations from urban air exposure varied from ~7-37 µg/m^3 in the Netherlands, Italy, the United Kingdom, and in the USA [3;7-9], with substantially higher levels (37-162 µg/m^3) reported in China [10]. In Norway, $PM_{2.5}$ levels in the largest cities have been in the range of 6-15 µg/m^3 over the years 2003-2015 [11]. Evidence for an association between $PM_{2.5}$ levels ranging from 10-30 µg/m^3 and lung cancer is now accepted [1].

Long-term exposure to $PM_{2.5}$ was estimated to have caused 4.2 million deaths worldwide in 2015, and CVD (ischemic heart- and cerebrovascular diseases combined) and lung cancer accounted for 31.3% and 16.5%, respectively, of attributable deaths [5]. Furthermore, an increase in $PM_{2.5}$ level of 5 µg/m^3 was associated with an 13% increase in non-fatal acute coronary events [5]. Thus, exposure to PM from urban air poses a risk for adverse health effects, both in the general population and in workers employed in outdoor-stationed occupations such as police officers, traffic controllers, and drivers [1].

1.1.3 Occupational exposure to diesel exhaust

Particulate matter from incomplete combustion of diesel fuel is one of the main contributors to PM air pollution [1]. It has been estimated that three million workers in the European Union (EU) are occupationally exposed to diesel exhaust (DE), including diesel exhaust particles (DEP) [12]. Adverse health effects from exposure to DE/DEP in occupational settings where diesel powered equipment is used in enclosed areas, are of particularly great concern [13].

Epidemiological studies on long-term occupational exposure to DE/DEP have established associations with increased risk of CVD [14;15], chronic obstructive lung disease (COPD) [16], and lung cancer [17-19]. Results obtained from a nested case-control study in non-metal miners (The Diesel Exhaust in Miners study) supported a causal effect of exposure to DE/DEP on lung cancer mortality [17]. Furthermore, results from a cohort study (also part of The Diesel Exhaust in Miners study) indicated a higher risk of lung cancer mortality in the group of ever-underground workers who experienced the highest levels of DE/DEP exposure [18]. A retrospective cohort study from trucking industry workers reported an increased association with cumulative exposure to DE/DEP and lung cancer mortality [19].

1.1.4 Classification of DE/DEP exposure as carcinogenic to humans

In 2012, DE including DEP, was classified by the International Agency for Research on Cancer (IARC) as "Carcinogenic to humans" (Group 1) [20], largely based on evidence of increased risk of lung cancer from studies in non-metal miners [17;18] and truck drivers [19]. Data supporting this classification predominantly relates to exposures from diesel engines produced before the early 2000s. Because of increasingly stricter emission regulations for both on- and off-road diesel engine vehicles, novel technology has emerged, gradually changing the mass and composition of the exhaust [21;22]. Thus, diesel engines may generally be divided into traditional- (pre-1989), transitional (~1989-2006), and new technology (2007 and later) engines (NTDE) [22].

The main studies contributing to the classification of DE/DEP as lung carcinogenic were debated [23-25]. Thus, both The Diesel Exhaust in Miners Study (DEMS) [17;18], and The Trucking Industry Particle Study [19], were reviewed by the Health Effects Institute (HEI). Furthermore, HEI also reviewed a study by Vermeulen *et al.*, (2014) which suggested an exposure-response curve (ECR) based on the same studies [26;27]. It was concluded that the studies were of high scientific quality and suitable for conducting quantitative risk assessments (QRA), but that the suggested ECR needed adjustment considering NTDE [26].

1.1.5 Composition of DE/DEP

Exhaust from diesel engines consists of a complex mixture of gases, volatile organic compounds, and particulate matter. The gas phase mainly includes oxides of carbon and nitrogen (CO_x, NO_x), in addition to low-molecular weight hydrocarbons, while particulate matter is composed of elemental carbon (EC) cores with adsorbed polycyclic aromatic hydrocarbons (PAH), nitro-PAH, a small amount of sulphate and nitrate compounds, and trace amounts of metals [28]. The particulate matter phase consists of both fine (<2.5 μm) and

ultrafine (<0.1 μm) agglomerates of particles [29]. Traditionally, EC is regarded as the best proxy indicator for measurement of DE/DEP exposure [30]. The chemical characteristics of DE/DEP are dependent on engine type, diesel composition, operating conditions, and applied exhaust after-treatments [29;31]. Hence, toxic effects of DE/DEP are largely affected by their inherent chemical characteristics. The mass and composition of DE/DEP have changed over the last three decades resulting in reduced levels of EC and PAH, aromatics and aldehydes [21;22]. Thus, the changing characteristics of DE/DEP from old versus newer technologies are of importance when considering the biological effects of exposure. Furthermore, the diesel fuel composition has changed over the years, affecting content of both sulphur and aromatic compounds [32]. The subject of biodiesel, however, is outside the scope of this thesis.

1.1.5.1 Combustion derived formation of PAH

PAH are formed by incomplete combustion and pyrolysis of organic matter and major anthropogenic sources are heating and cooking with coal or wood, DE/DEP, industrial emissions, diet, and tobacco smoke [33]. Several PAH are known or suspected carcinogens to humans, and PAH exposure constitutes increased risk of adverse health effects, both in the public and in occupational settings [33;34]. PAH are lipophilic compounds comprised of at least two aromatic rings containing solely hydrogen and carbon [35]. Atmospheric PAH of two or three rings are mainly present in the gas phase, whereas larger ringed PAH are adsorbed to DEP and/or PM [36]. Important combustion-related PAH are fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, B[*a*]P, ideno(123cd)pyrene, and benzo[ghi]perylene. The ratio of these combustion-related PAH and total PAH present in the atmosphere has been suggested to be used as an indicator of vehicle generated PM [37]. Nitrated PAH (nitro-PAH) and oxygenated PAH (oxy-PAH) can be generated both during the combustion process and from secondary reactions with primary PAH and atmospheric oxidants [38]. 1-nitropyrene is considered a relevant marker of DE/DEP exposure [39].

1.1.6 Occupational DE/DEP exposure levels

Several research groups have measured levels of combustion derived EC in various working environments where diesel powered equipment is used, including i.e. mining, tunnel construction, vehicle mechanics, and truck- and bus drivers [13]. EC air concentrations ranged from high levels (27–658 μg/m^3) measured in enclosed environments associated with mining and tunnelling, to intermediate levels (<50 μg/m^3) in above-ground semi-enclosed environments such as vehicle mechanics, warehouses etc., to low levels (<25 μg/m^3) measured

in outside work, or work separated from the exposure source [13]. In a Swedish study, EC air concentrations of 86.7 µg/m^3 for road tunnel finishing work, ~10.0 µg/m^3 for garage workers, and ~6.5 µg/m^3 for professional drivers was reported [40]. A Norwegian study recently reported that EC air concentrations ranged from: 45.5 µg/m^3 in aluminium smelters, 18.8 µg/m^3 and 1.2 µg/m^3 in underground and open-pit mining, respectively, and 2.7 µg/m^3 during baggage handling at an international airport [41]. EC exposure levels of 14 µg/m^3 for surface miners, 18-44 µg/m^3 for different underground occupation groups, and 59 µg/m^3 for underground diesel loader operators were measured in an Australian study [42].

1.1.7 DE/DEP exposure levels during tunnel construction in Norway

Since the early 2000s, the occupational exposures to DE/DEP during Norwegian tunnel constructions have improved from when the measured geometric mean (GM) EC levels during drilling and blasting were 160–340 µg/m^3 [43]. Still, recent exposure measurements indicate relatively high levels of GM EC, ranging from 31–54 µg/m^3 for different job groups participating in tunnel construction [44]. Exposure measurements during tunnel finishing work have only recently been conducted. Personal full-shift air-samplers were obtained from three tunnels during finishing work, showing GM EC concentration of 37.8 µg/m^3 [41].

1.1.8 Emission regulations for off-road diesel engines

Diesel engine emissions are strictly regulated in many countries worldwide. In Europe, off-road diesel engines are subjected to Stage standards. The aim is to reduce both gaseous and PM emissions and regulations in relation to fuel consumption at full load (g/kWh) and net engine power (kW). Stage I and II standards were implemented in the early 2000s. This was the first step in governmental emission regulation for off-road diesel run equipment [45;46]. With Stage III (2006-2013), PM emissions could not exceed 0.025 g/kWh [46]. Stage IV was implemented in 2014 with stringent reductions in NO_X (to 0.4 g/kWh) [46]. To fulfil the Stage standards, diesel particulate filter (DPF) and/or in-cylinder technologies such as catalysts are needed. However, all standards apply only for new vehicles and equipment [46]. In Norway, the Norwegian Environment Agency is expecting that emissions will remain constant to 2024 due to increased activities [47]. Stage V regulations will be effective in the next few years (2019 and 2020), and include adopting particle number (PN) emission limits ensuring use of wall-flow particulate filters on all affected engine categories, and reducing PM emission from 0.025 g/kWh to 0.015 g/kWh [46].

1.1.9 Occupational exposure limits to DE/DEP

As DE/DEP comprise a complex and highly variable mixture of constituents, the Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals (NEG) has together with the Dutch Expert Committee on Occupational Safety (DECOS) proposed to set OEL for DE/DEP both as EC and as NO_2 to cover the variability of diesel exhaust composition [48]. Few countries have established an OEL for DE/DEP or associated constituents. Both Austria and New Zealand have an eight hour EC OEL of 0.1 mg/m^3, whereas in Germany it is 0.05 mg/m^3 [49]. In Ireland and Poland, DE/DEP measured as respirable dust has an OEL of 0.15 mg/m^3 (particles<0.1 μm) and 0.5 mg/m^3, respectively [49]. In Sweden, OEL is set to 2.0 mg/m^3 NO_2 as a proxy for DE/DEP, and content of hydrocarbons in the air originating from diesel to 350 mg/m^3 (time weighted average for a working day) [50]. The OEL for NO_2 in Norway independent of exposure source is 0.96 mg/m^3 [51]. A QRA was recently published based on a revised ECR reporting a maximum tolerable risk of combustion derived EC of ~1.0 μg/m^3 [27;52]. EC levels measured in various occupations frequently exceed this level.

1.2 Mechanisms of toxicity of DE/DEP

1.2.1 Inhalation

NO_2 in the gas phase and DEP may constitute some of the most important factors when considering adverse health effects following DE/DEP exposure [4]. NO_2 is effectively absorbed (70–90%) in human airways after inhalation and may cause pro-inflammatory responses and oxidative stress [53;54]. Due to their small size, DEP deposit mainly in the peripheral parts of the lung where they may directly affect alveolar macrophages and epithelial cells of the alveoli [55;56]. *In vitro*, DEP are shown to initiate harmful responses through inflammation by increased cytokine/chemokine production and reactive oxygen species (ROS) formation, which may subsequently lead to DNA damage [55;57]. DEP (or DEP adsorbents) may also enter the bloodstream, eliciting systemic inflammation and reacting with blood constituents [58]. A brief overview of mechanisms of toxicity of DE/DEP is outlined in Figure 1.

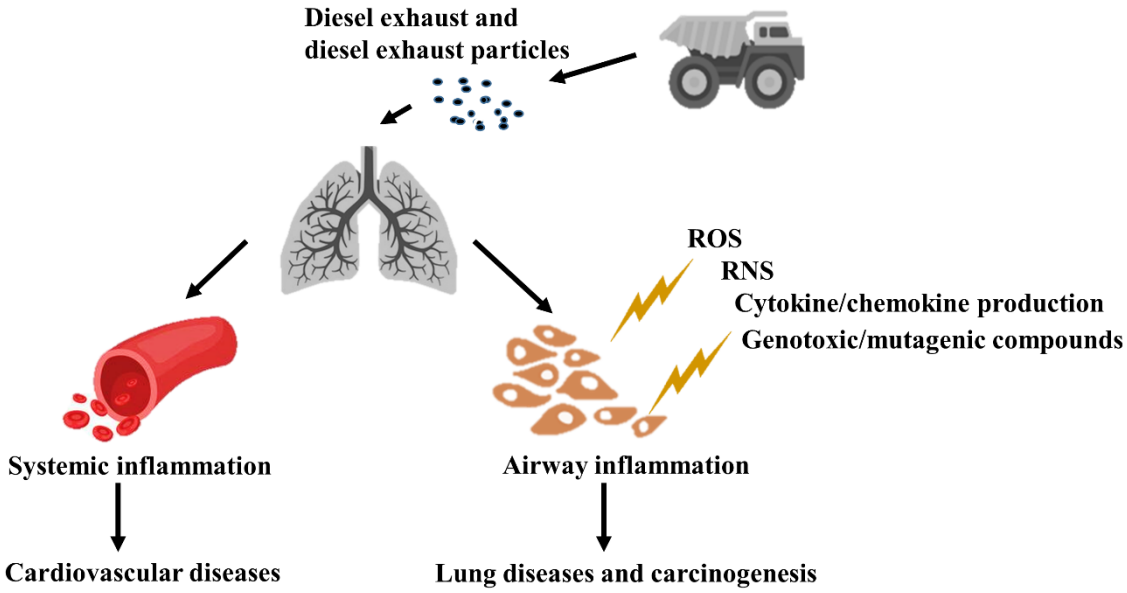

Figure 1. The figure displays a brief schematic overview of mechanisms of toxicity of inhaled diesel exhaust and diesel exhaust particles. ROS, reactive oxygen species; RNS, reactive nitrogen species.

1.2.2 Biotransformation of PAH and nitroarenes

PAH and nitroarenes present on DEP are important chemical carcinogens [20]. Through the aryl hydrocarbon receptor (AHR) pathway, PAH induce the cytochrome P450 monooxygenases CYP1A1/B1, which are key metabolising enzymes in formation of carcinogenic diol-epoxides, radical cations, and *o*-quinones [59]. Other routes of PAH biotransformation are the CYP peroxidase pathway and the AHR independent aldo-keto reductases (AKR) pathway [60]. PAH derivatives found in DEP, such as nitro-PAH and oxy-PAH, have received increasing interest as they may exert cytotoxicity, immunotoxicity, and carcinogenicity [20]. Nitro-PAH may be biotransformed to reactive metabolites by nitro-reduction and conjugation reactions through several different pathways [36]. A major pathway involves reduction of nitro-groups to arylamines, followed by N-hydroxylation and esterification [61]. Highly reactive metabolites can directly affect DNA, proteins, and lipids, and initiate carcinogenesis [59].

1.2.2.1 Cross-talk between the oestrogen receptor (ER)- and the AHR pathways

Emerging evidence from both epidemiological and experimental studies indicate that steroid hormones may have a role in lung carcinogenesis [62]. Cross talk between the oestrogen receptor- (ER) and the AHR pathway exists, and activation of ER may significantly affect AHR regulated genes [63;64]. Interestingly, lung tissue and lung cancer cell lines obtained from women express higher levels of *CYP1A1* compared to those obtained from men [65]. A highly

significant correlation between expression of *CYP1A1* and the level of DNA adducts in lung tissue has been reported [66]. Both *CYP1A1* and *CYP1B1* have the ability to hydroxylate 17β-oestradiol (E2), yielding 2-OH-E2 and 4-OH-E2, respectively [67]. 4-OH-E2 is genotoxic and may lead to DNA adduct formation [67]. In addition, *CYP1B1* may be regulated by E2 via oestrogen receptor alpha (ERα) [68]. Several 4- and 5- ring PAH share structural similarities with E2, and e.g. 3-methylcholantrene present in DEP is shown to directly activate ERα [69]. This indicates that *CYP1B1* may also be induced by non-AHR mediated mechanisms.

1.2.3 Generation of DNA adducts as an effect marker of DE/DEP exposure

DNA adducts are covalent bonds between a carcinogen/pro-carcinogen and DNA. Bulky DNA adducts are validated biomarkers of early effects related to PAH exposure [70], although the levels are influenced by an individual's xenobiotic metabolising abilities and DNA repair capacity [71;72]. If DNA adducts are not repaired, mutations may occur because of nucleotide substitutions or deletions, or chromosome rearrangements, initiating carcinogenesis [71].

Studying DNA adduct formation in lung tissue from healthy humans is difficult. Therefore, white blood cells (WBC), peripheral blood mononuclear cells (PBMC), or lymphocytes are frequently utilised as surrogates for bronchial lung cells. Previous studies have shown significantly increased levels of bulky DNA adducts in lymphocytes from urban area bus drivers and mechanics, truck terminal workers, and aluminium production plant workers who all may experience exposure to DEP [73-75]. Although DNA adduct formation correlates with PAH exposure, a non-linear relationship between high levels of PAH exposure and DNA adducts in WBC has been reported [76;77]. This discrepancy may probably be related to enzymatic saturation and/or induction of repair mechanisms [76; 77]. Furthermore, individual levels of DNA adducts are influenced by external factors other than carcinogenic occupational exposures. Smoking is shown to increase the levels of DNA adducts in PBMC compared with non-smokers [78;79]. A diet rich in fresh fruit and vegetables is shown to negatively affect the DNA adduct level in peripheral leukocytes [80]. It needs to be taken into considered that DNA adducts in surrogate tissues such as WBC, PBMC, or lymphocytes only indirectly reflect DNA adducts in the target organ. Nonetheless, measurements of DNA adducts from occupational exposures to PAH are highly informative when investigating groups of workers [70;72;81]. Recently, bronchial DNA adduct levels were shown to significantly correlate with increased risk of lung cancer development in a meta-analysis [82].

1.3 Acute and chronic health effects of exposure to DE/DEP

1.3.1 Acute health effects

Exposure to DE/DEP is associated with several acute health effects such as irritant effects of the eyes, throat and bronchia, headache, nausea, and cardiovascular effects, in addition to exacerbations of respiratory diseases and allergies [4;45]. Although debated, studies have suggested independent acute health effects of NO_2 on respiratory and cardiovascular symptoms when correcting for co-pollutants [83;84].

A study in healthy subjects exposed to 100 μg DEP/m^3 (including 0.2 ppm NO_2 and 0.04 mg/m^3 formaldehyde) for 2 h reported irritation in the nose, throat and eyes [85]. Healthy volunteers exposed to diesel exhaust (300 μg/m^3 for 1 h) experienced decreased peak expiratory flow (PEF), increased serum interleukin-6 inflammatory marker, in addition to self-reported throat irritation [86]. An increased bronchial, but not alveolar response was observed in healthy subjects exposed to 100 μg DEP/m^3 (2 h) [87]. A study in male patients with stable coronary heart disease exposed to diesel exhaust (300 μg/m^3 for 1 h) showed increased myocardial ischemia and inhibition of endogenous fibrinolytic capacity [88]. A similar exposure study reported systemic and pulmonary inflammatory responses in healthy volunteers, whereas no changes were measured on standard lung function parameters such as PEF, forced expiratory volume during the first second (FEV1), and forced vital capacity (FVC) [89]. Thus, health assessments relying solely on lung function parameters may underestimate exposure related biological effects. Gene expression profiling of PBMC following controlled human exposure to DE/DEP, has revealed upregulation of genes involved in e.g. oxidative stress, inflammation and mitochondrial dysfunction [90;91].

1.3.2 Cardiovascular diseases; epidemiology and aetiology

Cardiovascular diseases (CVD) comprise diseases of the heart and blood vessels, and is the number one cause of death worldwide (~30%) with an estimated >400 million prevalent cases in 2015 [92]. The age-standardized CVD death rate has declined between 1990-2015 in high-income and some middle-income countries, although relatively unchanged levels were observed for most regions [92]. Exposure to particulate air pollution and diesel exhaust constitutes some of the main risk factors for developing CVD [92]. Air pollution is associated with increased risk of endothelial dysfunction and vasoconstriction, increased blood pressure (BP), pro-thrombotic and coagulant changes, systemic inflammation and oxidative stress responses, autonomic imbalance and arrhythmias, as well as atherosclerotic development [6;93]. An increased relative risk of ischemic heart disease following occupational exposure to

DE/DEP was reported in Swedish male construction workers [14]. A Swedish population based case-control study found a moderately increased risk for myocardial infarction in occupationally DE/DEP exposed workers [15].

1.3.3 Chronic obstructive pulmonary disease; epidemiology and aetiology

Chronic obstructive pulmonary disease (COPD) was in 2002 the fifth leading cause of deaths worldwide [94]. It has been estimated that by 2030, COPD will become the third leading cause of deaths worldwide [94]. The disease is characterised by chronic airway obstruction and includes conditions of emphysema and chronic bronchitis. COPD is largely a heterogenic disease diagnosed by a ratio of FEV1/FVC<0.7, and it is strongly related to lung cancer development [95;95;96]. Although tobacco smoking is the main cause of COPD development, it was estimated that 15-20% of COPD cases may be attributed to occupational exposures [97]. A case-control study reported a strong association between COPD and occupational exposure to DE/DEP with an odds ratio (OR) of 1.9 (95 % CI = 1.3, 3.0) [16].

1.3.4 Lung cancer; epidemiology and aetiology

Lung cancer is the leading type of cancer and cause of cancer-related deaths worldwide [98]. In Norway, lung cancer accounts for ~10% of newly diagnosed cancers, with approximately 3000 cases every year [99]. Tobacco smoking is the main aetiological factor for developing lung cancer, but several other environmental factors may also increase disease risk [100]. It has been reported that between 7.9–16.5% and 1.4–4.05% of lung cancer cases in males and females, respectively, in the EU may be attributed to occupational exposures [101]. In Norway, 1 out of 5 lung cancer cases in men were estimated to be work-related [102]. The five-year lung cancer survival rate (~20%) is low, although increasing, highlighting the need for both better diagnostic tools and treatment options [99;103].

1.3.4.1 Tobacco smoking and lung cancer

Lung cancer trends and incidence rates are highly influenced by age, sex, ethnicity, socioeconomic status, and geography; which is mainly due to differences in onset and cessation of tobacco smoking [100]. The first preliminary report of an association between cigarette smoking and lung cancer was published already in 1950 [104]. In several Western countries, lung cancer age-adjusted rates have now peaked for men, with a general decline in incidence rates since the early 1990s [99;100;103]. In most countries, lung cancer incidence rates for women have not peaked, reflecting the later adaption of smoking habits in women as compared to men [99;100;103]. Although the global age-standardized prevalence of daily smokers is declining due to tobacco control policies, this net reduction constitutes a decrease in high-

income countries and an increase in low-income countries [100]. Thus, in many low- and middle income countries the lung cancer epidemic following tobacco smoking has not yet begun [100].

1.3.4.2 Lung cancer histology

Lung cancer is divided into two main groups: non-small cell lung cancers (NSCLC) and small-cell lung cancers (SCLC). Approximately 85% of diagnosed lung cancer cases are NSCLC, constituting adenocarcinoma (ADCA), squamous cell carcinoma (SCC), and the heterogeneous group of large-cell carcinoma (LCC) [105]. SCLC is among the most aggressive tumours with high proliferation rates and early onset of metastasis [105]. In line with changes towards filter/low-tar cigarettes, the histopathology of lung cancer cases have shifted from mainly SCC and SCLC, being central tumours in the lung, to mainly peripheral tumours of ADCA, and LCC [106]. ADCA is also the subtype most frequently found in women and in never-smokers [107]. Furthermore, exposure to particulate air pollution may be stronger associated with ADCA than with other histological subtypes [108;109]. However, a recent Swedish case-control study investigating long-term occupational exposure to DE/DEP reported an association between the exposure, lung cancer risk, and particularly SCC and undifferentiated, large cell, anaplastic or mixed carcinomas [110].

1.4 Lung carcinogenesis

1.4.1 Hallmarks of cancer

Cancer covers a wide range of diseases characterised by uncontrolled growth of cells. Several hallmarks of cancer are proposed enabling tumour growth and metastasis [111] (Figure 2). Clonal evolution selects for neoplastic cells with increased proliferation and survival rates by activation of growth promoting and inhibition of tumour suppressor pathways, which may lead to invasive and metastatic capacities [112]. By mutations and epigenetic changes, cancers acquire various phenotypes, thus continuously changing genotype, phenotype, and clonal structure over time [113].

Figure 2. The hallmarks of cancer. The figure depicts events in the transformation of normal cells to cancer cells. The emerging hallmarks are denoted by asterisks (*), whereas the enabling characteristics are marked with double asterisks (‡). Modified from [111].

1.4.2 Molecular aberrations in lung cancer

Lung cancer development inevitably involves genomic instability and the acquirement of mutations. However, compared to several other types of cancers, lung cancers show abundant genetic diversity with relatively few recurrent somatic mutations and genomic rearrangements occurring at high frequencies [114-116]. The following paragraphs briefly mention a few molecular aberrations with focus on NSCLC.

Analysis of ADCA cases by whole-exome sequencing identified 18 statistically significant mutated genes having roles as oncogenes (*KRAS* (33%), *EGFR* (14%), *BRAF* (10%), *PIK3CA* (7%), and *MET* (7%)), tumour suppressors (*TP53* (46%), *STK11* (17%), *KEAP1* (17%), *NF1* (11%), *RB1* (4%), and *CDKN2A* (4%)), small GTPases (*RIT1* (2%)), chromatin modifying genes (*SETD2*, *ARIDA1A*, and *SMARCA4*), and RNA splicing genes (*RBM10* (8%) and *U2AF1* (3%)) [115].

SCC is associated with complex genomic alterations and several genes have been reported to be recurrently mutated, among them oncogene *(PIK3CA)*, tumour suppressors (*TP53*, *CDKN2A*,) and histone methyl transferase *(MLL2)* [116;117]. Furthermore, somatic mutations in genes involved in oxidative stress responses (*KEAP1*, *CUL3*, and *NFE2L2*) and squamous

differentiation (*SOX2*, *TP63*, and *NOTCH1*) were frequently identified [116]. Combined, ADCA and SCC harbour both unique and common somatic mutations.

TP53 is an important tumour suppressor that responds to oncogenic cellular stressors, e.g. DNA damage, and induces expression of central downstream genes being involved in cell cycle arrest, DNA repair, and apoptosis [118]. To be noted, inactivation of *TP53* via mutations and loss of heterozygosity was reported to occur in 90% of SCLC and ~50% of NSCLC cases, respectively [119;120]. The association of *TP53* mutations with prognostic significance is not clear, however, there are indications of lower survival rates in early-stage NSCLC or ADCA patients with mutations in *TP53*, as compared to patients without such mutations [120].

Interestingly, mutations in *KRAS* occur frequently in ADCA (in 25-40% of cases) and particularly in daily-smoking patients from Western populations, but are rare or absent in SCC or SCLC [114;121]. *EGFR* is mutated in 10-15% of unselected Western patients and in 30-40% of Asian populations [114]. The geographical variability may partly be because *EGFR* mutations frequently occur in ADCA in younger, non-smoking females [122], reflecting the significant contribution of indoor air pollution from cooking and heating to the lung cancer burden particularly in Eastern Asia [100]. However, a recent study analysing cohorts of non-smoking adenocarcinoma cases in the USA and East Asia, did not find a significant sex-difference in prevalence of *EGFR* mutations [121]. Interestingly, *EGFR* mutations did not occur in tumours with *KRAS* mutations, and may define a distinct subset of pulmonary ADCA which is not caused by tobacco carcinogens [122].

Both *BRAF* and *MET* are mutated in 3-5% of mainly ADCA, and *MET* is also associated with high copy number gain in 4-6% of NSCLC [123]. Rearrangements of *ALK* (2-7%), *ROS1* (1-2%), and *RET* (1-2%) in NSCLC are mostly identified in ADCA of non- or light smoking younger patients, whereas *NTRK* rearrangements is identified in 2-3% NSCLC of any histological subtype and is not associated with age, sex, or smoking history [123].

1.4.3 Epigenetics in cancer

Epigenetic modifications of signal transduction may be as important as genetic background during carcinogenesis [124], and are strongly associated with environmental exposures [125]. The word epigenetic is derived from the Greek prefix *epi*, meaning upon, near to, in addition. Epigenetic regulation can be dynamic and reversible, and regulates gene expression without concomitant alterations of the DNA sequence. Epigenetic changes may also lead to reactivation of endogenous retro-elements, and to general genomic instability [111;126]. Epigenetic

modifications include incorporation of histone variants, covalent histone modifications, nucleosome re-positioning, DNA methylation, altered expression of non-coding RNA (ncRNA), and post-transcriptional modifications of RNA [126]. The following paragraph will focus on microRNA (miRNA).

1.4.3.1 miRNA biogenesis and regulation of gene expression

miRNA are evolutionary conserved, single-stranded, and small ncRNA (~22 nucleotides). miRNA is transcribed by RNA polymerase II forming hairpin primary-miRNA, which is cleaved by DROSHA-DGCR8 giving rise to precursor-miRNA. Precursor-miRNA is then exported to the cytoplasm by Exportin 5, and cleaved by DICER [127]. Distinct miRNA are generated either from the 3'- or the 5' arm of precursor-miRNA by the RNA-induced silencing complex (RISC) [128], and are often subjected to post-transcriptional modifications [127;129]. miRNA regulate ~30% of coding genes at the post-transcriptional level [126]. The "seed" region of miRNA is crucial for target recognition, and is the sequence spanning from position 2 to 8 at the 5' end [130]. This seed-sequence pairs fully with its response element in mRNA, which is mainly located at the 3' untranslated region, leading predominantly to destabilization and decay of mRNA and translational repression [131] (Figure 3). The biological significance of deregulated miRNA is generally complicated by that multiple mRNA may potentially be targeted by each miRNA, and that each mRNA may be targeted by several miRNA [128].

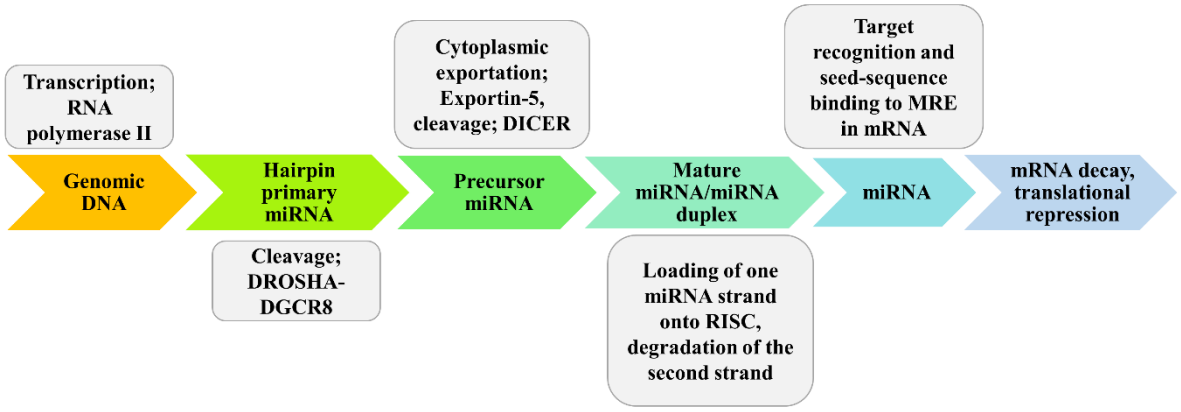

Figure 3. A simplified overview of miRNA biogenesis and regulation of gene expression (modified from [131]).

1.4.3.2 miRNA frequently deregulated in lung cancer

Different miRNA may be promising markers of diagnosis and prognosis of lung cancer, as they are present in both tissue and body fluids such as blood and sputum [132]. miRNA of the let-7 family are shown to regulate expression of RAS oncogenes [133], and are frequently reported

to be downregulated in lung cancer [134;135] which points towards a tumour suppressor function of let-7. In contrast, miR-155 is an important oncomir in lung carcinogenesis. High levels of miR-155 and low levels of let-7a-2 in primary NSCLC lung cancer tissue correlated with poor survival [135]. A negative feedback-mechanism has been reported linking expression of miR-155 and *TP53*. Thus, high miR-155 expression and low expression of *TP53* in NSCLC tissue was associated with shorter survival [136]. miR-21 is frequently upregulated in lung cancer, and was shown to be a downstream effector of the EGFR signalling pathway [137]. Expression of miR-21 and miR-155 were upregulated in NSCLC tissue samples, and both miRNA, alone and together, were shown to directly inhibit several tumour suppressor genes, including PTEN [138]. miR-205 is shown to promote growth and metastasis of NSCLC through downregulation of PTEN [139]. miR-205 may serve as a potential diagnostic marker of NSCLC [140]. Moreover, downregulation of miR-205 in A549 induced apoptosis and inhibited proliferation, invasion, and migration [140].

Analysis of cell-free miRNA in blood plasma from NSCLC patients revealed deregulation of fourteen miRNA (miR-19a-3p, miR-19b-3p, miR-130b-3p, mir-30e-5p, mR-486-5p, miR-22-3, miR-16-5p, miR-15b-3p, miR-29c-3p, miR-140-3p, miR-29b-3p, miR-210, miR-24-3p, miR-144-5p, miR-20a-5p, mR-222-3p, miR-150-5p, miR-425-5p, miR-324-5p, and miR-133a) compared with healthy controls [141]. As circulating miRNA may originate from different locations in the body, care must be taken when interpreting such results in relation to lung carcinogenesis. However, several of the above mentioned circulating miRNA are associated with malignant processes of the lung [141] and may thus turn out to represent valuable biomarkers.

1.4.3.3 Deregulated miRNA following exposure to air pollution or DE/DEP

Deregulation of several miRNA have been identified in response to air pollution and DE/DEP exposure. A recent review identified nine miRNA, miR-9, miR-10b, miR-21, miR-128, miR-143, miR-155, miR-222, miR-223, and miR-338, as deregulated in two or more independent air pollution studies [142]. *In vitro* DEP exposed (10 µg/cm^3 for 24 h) HBEC grown at air-liquid interface showed significant deregulation of 197 miRNA compared with control cells [143]. The twelve most deregulated miRNA (miR-513c, miR-513b, miR-513a, miR-923, miR-496, miR-338-5p, miR-31, miR-26b, miR-96, miR-27a, miR-135b, and miR-374a) were associated with inflammation and carcinogenesis [143]. miR-21, miR-31e, mir-215, and miR-144 were reported deregulated in PBMC isolated from DE/DEP exposed (300 µg $PM_{2.5}$/m^3 for 2 h) human volunteers [144]. Deregulation of several miRNA (miR-421, mir-146a, mir-29a, and let-

7g) affecting important inflammatory genes were identified in a study on foundry workers exposed to metal-rich PM [145]. Although substantial research efforts reporting changes in miRNA expression profiles following exposures to chemical carcinogens [142], the mechanistic role of miRNA in chemical-associated carcinogenesis remains relatively unexplored [131].

1.4.4 Epithelial-to-mesenchymal transition

Cancer cells of epithelial origin may acquire malignancy through epithelial-to-mesenchymal transition (EMT). Epithelial cells show apical-basal polarity and are connected in sheets to the basement membrane. Adherens junctions stabilize cell-cell adhesions, in addition to regulating the actin cytoskeleton, mediating intracellular signalling, and regulating gene expression [146]. These junctions are composed of transmembrane proteins belonging to the cadherin family, forming homodimers in the plasma membrane between interacting cells, in addition to intracellular anchor proteins such as catenin (i.e. β-catenin). Disruption of normal cell-cell adhesion may lead to malignant progression and tumour cell metastasis [147].

EMT and the reverse process of mesenchymal-to-epithelial transition are important events in developmental processes and tissue repair [148;149]. However, these processes can be reactivated in cancer cells enabling increased migration and invasive potential. As EMT/MET are reversible processes, a spectrum of multiple states of partial EMT between epithelial and mesenchymal phenotypes may be frequent in tumours [126]. Downregulation of E-cadherin (*CDH1*) and subsequent upregulation of N-cadherin (*CDH2*) (the so-called cadherin switch), is commonly observed during EMT [149]. Additionally, the mesenchymal marker vimentin (*VIM*) is upregulated ensuring cytoskeleton architecture and cellular mechanical strength [150].

Both genetic and epigenetic alterations participate in mediating the transition from an epithelial to a mesenchymal phenotype. The main EMT transcription factors are SNAI1/2, ZEB1/2, and TWIST1, which participate in repressing *CDH1* expression [151]. TWIST1 may also be involved in upregulating *CDH2* [152]. *CDH1* may be targeted by DNA methylation and it was shown that *CDH1* repression resulting from promoter hyper-methylation was associated with increased risk of lung cancer [153;154]. ZEB1 and ZEB2 can both be transcriptional activators and repressors by binding i.e. to histone acetyl transferases or histone deacetylases [155]. Emerging evidence shows that ZEB proteins are downstream targets of SNAI and TWIST1 [155]. In lung cancer cell lines, *ZEB1* is found to have a strong inverse correlation with *CHD1* expression [156;157]. Members of the miR-200 family (miR-200a, miR-200b, miR-200c, and miR-141) participate in maintaining an epithelial phenotype by inhibiting *ZEB1* and *ZEB2*.

Interestingly, these miRNA are transcriptionally repressed by ZEB and SNAI1 through a double-negative feedback loop [158]. Additionally, prostaglandin E synthase is shown to induce *ZEB1* and *SNAI1* in NSCLC cell lines with subsequent downregulation of *CDH1*, highlighting the complexity of EMT regulation [159;160]. Downregulation of *CDH1* render β-catenin free for nuclear translocation promoting β-catenin dependent gene expression of e.g. the Wnt signalling pathway, which has prominent roles in human malignancies [147].

1.5 Signalling eicosanoids in inflammation and cancer

1.5.1 Generation of eicosanoids from polyunsaturated fatty acids

Eicosanoids from enzymatic or non-enzymatic oxidation of polyunsaturated fatty acids (PUFA) are important signalling molecules mediating normal tissue homeostasis, inflammatory responses, and cancer progression [161;162] (Figure 3). As the eicosanoids may be either beneficial or detrimental depending on cellular or tissue context, the exact role of modulation of expression of these metabolites is difficult to elucidate [161;162].

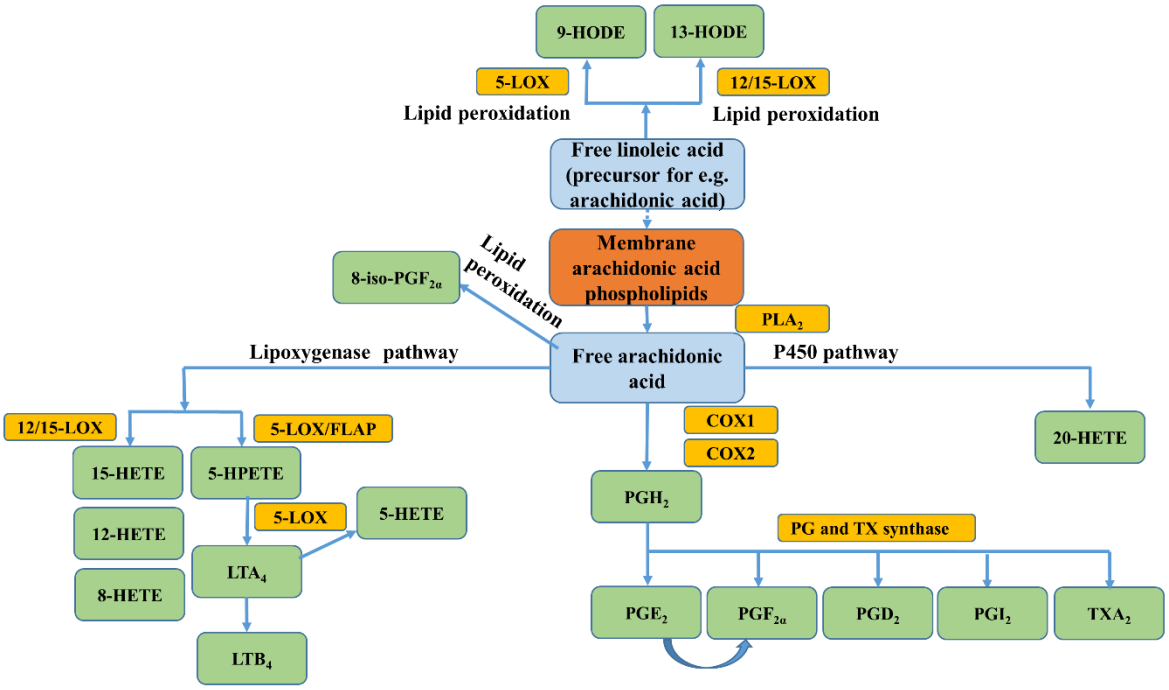

Figure 4. A simplified overview of eicosanoids derived from arachidonic acid (AA) and linoleic acid (LA). Eicosanoids in green, polyunsaturated fatty acids (PUFA) in blue, and enzymes in orange. Modified from [163;164].

1.5.2 Arachidonic acid metabolism

Arachidonic acid (AA) metabolism gives rise to prostaglandin (PG), thromboxane (TXA_2) lipoxine (LX), leukotriene (LT), in addition to hydroxyeicosatetranoic acid (HETE) and epoxyeicosatrienoic acid metabolites. AA is freed from the cellular membrane in a process catalysed by phospholipase A_2 (PLA_2) enzymes which specifically hydrolyse the *sn-2* position of phospholipids [165]. The cytosolic calcium (Ca^{2+}) dependent PLA_2 ($cPLA_2$) preferentially hydrolyses AA [165]. Higher levels of free AA due to altered expression of $cPLA_2$ has been found in lung tumours from mice harbouring an activated *MYC* gene [166]. Free AA is substrate for oxidation by either cyclooxygenase (COX) 1 or 2, lipoxygenase (LOX), or cytochrome P450 (CYP) pathways. Non-enzymatic lipid peroxidation of AA may form isoprostanes like 8-iso-$PGF_{2\alpha}$, which is a marker of oxidative stress [167]. Plasma lipid peroxidation products are reported to be suitable markers of biologically effective dose of PM exposure reflecting airway oxidative stress [168].

The COX pathway yields various PG and thromboxane by induction of different PG synthases [161]. The role of *COX-2* and PGE_2 in inflammation and cancer is well documented [161]. However, PGE_2 is also reported to participate in resolving inflammatory responses through amplifying LX expression, in addition to inhibiting *COX-2* expression [169]. Thus, PGE_2 may play a diverse role in carcinogenesis. Non-steroidal anti-inflammatory drugs inhibiting expression of *COX-2* has been reported to reduce the risk of lung cancer, although resulting in increased risk of cardiovascular diseases [170]. PGD_2 is generally reported as having anti-tumorigenic functions [164].

Generation of 15-HETE through the LOX pathway participates in the pathogenesis of hypertension and cardiac hypertrophy [171]. Moreover, 15-HETE signalling is suggested to have a role in platelet activation and pulmonary vascular thrombosis [172], whereas 20-HETE, generated through the CYP pathway, may elicit vasoconstrictor effects on small arteries and arterioles [173]. Increased levels of 5-, 12-, and 15-HETE may have roles in cancer cell growth, adhesion, migration, and invasion, in addition to angiogenesis [174].

1.5.3 Linoleic acid metabolism

Linoleic acid (LA) is an essential PUFA, an AA precursor, and it is a major constituent of low-density lipoproteins [162]. LA is prone to oxidation by endogenous enzymes in the circulation in addition to ROS, yielding different derivatives including hydroxyoctadecadienoic acids (HODE) [162]. HODE metabolites are among the most abundant lipid oxidation products measured in human plasma [175], and 9- and 13-HODE metabolites are shown to be the most

predominant derivatives of LA having implications for inflammatory regulations [162]. Furthermore, a role for lipid peroxidation in mediating PM-induced atherosclerosis has been suggested [176].

2 AIMS OF STUDY

The overall objective of this project was to investigate biological effects of exposure to DE/DEP *in vivo* and *in vitro*, in addition to comparing the effects of DEP exposure with those of CSC and B[*a*]P at the cellular level.

To achieve this, the project studied:

- Biological effect markers in a DE/DEP exposed occupational group.
- Toxic mechanisms using *in vitro* human bronchial epithelial cell models.

More specifically, the project investigated:

- The biological impact of occupational exposure to DE/DEP in Norwegian tunnel finishing workers.
- The potential of DEP to transform a HBEC line *in vitro* and to assess differences in sensitivity to DEP in untransformed and transformed HBEC lines.
- Molecular alterations in HBEC lines chemically transformed by DEP, CSC, or B[*a*]P.

3 SUMMARY OF PAPERS

Paper 1: Bulky DNA adducts, microRNA profiles, and lipid biomarkers in Norwegian tunnel finishing workers occupationally exposed to diesel exhaust

Exposure to diesel exhaust may cause lung cancer in humans. Tunnel finishing workers (TFW) are fitters performing work related to electrical installations, rock support, and water- and frost protection in road and railway tunnels. Despite changes in diesel technology and stringent emission restrictions, workers engaged in tunnel finishing work in Norway are exposed to high levels of DE/DEP from heavy-duty diesel powered equipment. The biological impact and potential adverse health effects associated with tunnel finishing work is not clear. This study reported that TFW had significantly higher levels of bulky DNA adducts in peripheral blood mononuclear cells (PBMC) compared with a referent group. Small RNA sequencing indicated that several miRNA related to apoptosis, antioxidant effects, and carcinogenesis were deregulated in PBMC in TFW. Subsequent pathway analysis indicated potential deregulation of pathways associated with cancer, alterations in lipid molecules, steroid biosynthesis, and cell cycle. Measurements of free arachidonic acid (AA) and eicosanoids showed altered plasma profiles in TFW compared with referents, with higher levels of AA and 15-HETE and lower levels of PGD_2 and 9-HODE in the DE/DEP exposed workers. Although the carcinogenicity of diesel exhaust is debated, results obtained in this study indicated that tunnel finishing work may be associated with an increased risk of adverse health effects. This study may have implications for risk assessment and preventive strategies concerning occupational exposure to DE/DEP.

Paper 2: *In vitro* transformation of human bronchial epithelial cells by diesel exhaust particles: gene expression profiling and early toxic responses

The human bronchial epithelial cell line HBEC3 was exposed to diesel exhaust particles (DEP) for 15 weeks in an *in vitro* transformation assay. Long-term DEP exposed HBEC3 formed significantly more colonies in soft agar compared to unexposed control cells. Four DEP transformed clones were established in monolayer culture and screened for epithelial-to-mesenchymal-transition (EMT) marker genes. All clones showed reduced, although variable, expression of *CDH1*. Generally, variability in other EMT marker genes were found. Based on this initial screening, one clone (T2-HBEC3) was subjected to further studies. Besides reduced *CDH1* expression, upregulation of *CDH2, VIM,* and several EMT transcription factors were measured in T2-HBEC3. Additionally, T2-HBEC3 displayed a mesenchymal/fibroblast-like morphology, reduced migration compared with parental HBEC3, and little invasive capacity.

Baseline differences between parental HBEC3 and T2-HBEC3 were explored by gene expression profiling. Results revealed deregulation of genes involved in lung carcinogenesis. Next, to investigate differences in sensitivity to DEP between HBEC3 and T2-HBEC3, short-term exposure experiments were performed. Gene expression profiling revealed deregulated expression of genes involved in metabolism of xenobiotics, lipids, and inflammation, all having implications for carcinogenesis. HBEC3 showed a higher steady state of *IL1B* expression and IL-1β secretion compared with T2-HBEC3. Expression of *CYP1A1* was higher and induced at an earlier time-point in T2-HBEC3 compared to HBEC3 indicating a greater xenobiotic response to DEP in the transformed cells. Combined, this inverse association between induction of CYP enzymes and inflammation may represent a mechanism to increase cell survival following DEP exposure. Interestingly, similar susceptibility to DEP induced genotoxic effects were found in both cell lines. Eicosanoid secretion was measured to further investigate differences in sensitivity to DEP between HBEC3 and T2-HBEC3. Generally, higher levels of major prostaglandin species were measured in cell culture media from T2-HBEC3 compared with HBEC3 and several metabolites were affected following the exposures. Together, the results showed that DEP have the potential to transform HBEC3 *in vitro*. Differences between parental HBEC3 and T2-HBEC3 regarding steady state levels and DEP induced changes of particularly *CYP1A1,* IL-1β, PGE_2, and $PGF_{2\alpha}$ may have implications for acute inflammation and carcinogenesis.

Paper 3: Gene and microRNA expression profiles of human bronchial epithelial cells transformed *in vitro* by diesel exhaust particles, cigarette smoke condensate, or benzo[*a*]pyrene

Lung cancer is the most frequently diagnosed cancer and cause of cancer-related deaths worldwide. Although tobacco smoking is the main aetiological factor for developing cancer of the lung, environmental and occupational exposures to air pollution, diesel exhaust, and PAH compounds, comprise additional important risks. Transformed cell lines developed from long-term exposures of parental human bronchial epithelial cell (HBEC) lines to the chemically complex cigarette smoke condensate (CSC), diesel exhaust particles (DEP), or benzo[a]pyrene have previously been established. Although the transformed cell lines commonly displayed traits of epithelial-to-mesenchymal transitions (EMT), differences existed between the cell lines. In order to further investigate mechanisms behind the chemically induced *in vitro* transformation process, gene expression profiling and small RNA sequencing were performed on clones of the different transformed cell lines. The aim was to identify deregulated genes and

miRNA at the basal level and associated deregulated signalling pathway. Results indicate the importance of genes and miRNA affecting carcinogenesis, EMT, and the extracellular matrix. Furthermore, alterations in the aryl hydrocarbon receptor pathway and inflammatory responses were identified. In conclusion, both general and specific changes in gene- and miRNA expression profiles were identified in the transformed cell lines. Combined, this study may increase the understanding of molecular events in the process of chemical induced *in vitro* transformation of human bronchial epithelial cells.

4 DISCUSSION

4.1 Methodological considerations

Detailed information regarding materials and methods is found in each respective paper (I-III). A brief discussion of selected methods and statistical considerations is presented here as to support the general discussion of the main results.

4.1.1 Peripheral blood mononuclear cells as surrogate tissue

PBMC have been reported as non-invasive surrogates for PAH exposed lung tissue [177;178]. While PBMC constitute different cell types with a round nucleus (i.e. lymphocytes, monocytes, and dendritic cells) related to immune responses, one cannot exclude the possibility that differences in PBMC composition between individuals may be of importance. Multiple physiological factors such as nutritional status, hormone levels, and infections/inflammation may influence the reactivity and thus the composition of PBMC, potentially leading to increased inter-individual variation [179]. In **paper I**, the composition of PBMC from each participant was not investigated. Several other studies using PBMC have also not differentiated on the various cell subsets [177;178]. Short-term exposure to air-pollution may be associated with changes in the WBC count in healthy subjects [180], and total lymphocyte count including levels of T-cells and B-cell subsets were reported to be higher in Chinese workers occupationally exposed to DE/DEP when compared to a reference group [181]. However, several other studies report no effects on circulating WBC counts after exposure to diesel exhaust [182;183]. Furthermore, levels of a potential inflammatory marker related to DE/DEP exposure was independent of blood cell counts [184]. The main objective of **paper 1** was to investigate biological alterations in TFW following occupational DE/DEP exposure compared to a similar referent group. There were no obvious indications of differences in health status between the two groups, which is in support of using the whole PBMC pool as a model system.

4.1.2 Study population

Personal EC exposure measurements from the TFW investigated in **paper I** were not performed. However, measurements from the breathing zone during tunnel finishing work in three Norwegian construction companies have recently been conducted [41]. TFW constitutes a mobile workforce, and these measurements were representative for the exposure scenarios of the present study.

Possible confounding due to healthy worker effect is of concern in occupational studies [185]. Here, all study participants were employed at the time of enrolment. Women were excluded

because of a low number of potential participants, in addition to a stronger healthy worker effect reported in women than men [185]. In general, the study participants have similar socioeconomic status. The study group was composed of all Caucasian males and the results could possibly have limited relevance in other populations, including Caucasian females. In addition, the cross-sectional study design combined with the one blood sample obtained from each participant without follow-up, could lead to incidental results. However, the grouping of TFW and referents without major stratification limits the potential influence by such bias. Referents constitutes a more heterogeneous group as compared to the TFW. Referents working at tunnel construction sites are occasionally inside tunnels for shorter time-periods. Also, older referents have in many cases a previous work record as TFW. In addition, one cannot exclude the possibility of potential minor chemical exposures for outdoor road workers from asphalt, vehicle emissions, and chemicals used for graffiti-removal. Thus, this could potentially participate in underestimations of the measured DNA adduct levels in TFW compared with referents.

BMI was used as a potential effect modifier for differences in diet. Analyses of human samples may be complicated by the heterogeneity of age, sex, and lifestyle factors. It is difficult to correct for every possible effect modification, but age, sex, smoking, and diet (BMI) may be important factors in metabolomics analysis [186]. It would have been beneficial to obtain the blood samples after fasting, but that was unfortunately not possible to implement. Also, intake of dietary supplements like fish-oil (or fish-oil capsules) could have been included in the questionnaire. Physiological rhythm may possibly affect the metabolome [187]. Although the majority of blood samples were taken during the afternoon, alternating teams worked day/night shifts at the time of sampling, and data are missing as to which study participant worked which shift.

4.1.3 ^{32}P-postlabelling

Monitoring bulky DNA adduct formation in human samples is an important component in studies on hazard identification for carcinogens and mutagens [188]. In **paper I**, the ^{32}P-postlabelling method was selected to measure levels of bulky DNA adducts following occupational exposure to DE/DEP. Briefly, in the ^{32}P-postlabelling assay, adducts are radioactively labelled after digestion of DNA to nucleoside 3'-monophosphates, followed by thin-layer chromatography (TLC) [188]. Next, total bulky DNA adduct levels derived from both PAH and nitro-PAH exposure can be measured in the diagonal radioactive zone (DRZ) area of the TLC-plates [188]. This method offers high sensitivity. Although the ^{32}P-

postlabelling assay does not specifically determine the chemical species responsible for the DNA adducts detected, the assay provides an estimation of the adduct-forming capacity of the environmental exposure in question. Other methods could have been used, such as immunoassay with antibodies for a selected range of bulky DNA adducts, gas chromatography, high-performance liquid chromatography, fluorescence spectroscopy, or mass spectrometry (MS) based methods [189]. MS has the advantage of high chemical specificity, but require expensive equipment, and large quantities of DNA. Moreover, MS-based methods have not yet been successful in detecting nitro-PAH specific DNA adducts in human specimens. However, a novel method for source-specific haemoglobin adducts derived from nitro-PAH exposure was recently published [190]. In this project, air contamination during tunnel finishing work is predominantly due to exposure from diesel powered equipment. Thus, the higher measured level of bulky DNA adducts in TFW is mainly related to PAH/nitro-PAH exposure from DE/DEP.

4.1.4 In vitro cell culture

In vitro cell culture (**paper II and III**) are highly simplified models designed to represent *in vivo* conditions. Compared to cell lines of cancer origin or viral immortalization, the human bronchial epithelial cell (HBEC) lines used in this thesis represent a particularly well suited model to study *in vitro* cell transformation [191;192]. HBEC lines are immortalised by transfection of retroviral constructs with the two human genes *hTERT* and *CDK4* preventing telomere-dependent senescence and premature growth arrest [191]. Importantly, HBEC lines show an epithelial morphology, cluster with primary HBECs distantly from cancer cell lines (i.e. A549) and cell lines immortalised by viral transfection (e.g. BEAS-2B) after gene expression profiling, have retained the ability to differentiate, have a stable genome, and an intact TP53 checkpoint pathway [191]. Moreover, these cells show low levels of spontaneous transformation [191;193;194]. Thus, HBEC lines are considered particular relevant models for *in vitro* carcinogenesis studies [191;195].

4.1.5 Liquid chromatography-tandem mass spectrometry

In **paper I,** plasma was isolated from the human blood samples and in **paper II,** cell culture media were collected from HBEC3 and T2-HBEC3 in the short-term DEP exposure experiments. Both plasma and cell culture media were analysed for circulating free AA and eicosanoids. Briefly, solid-phase extraction was used for AA metabolites from plasma and cell culture media followed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Eicosanoids have relatively low abundance. The LC/MS/MS method was chosen since it has

been considered better suited for analyses of eicosanoids than e.g. immunoassay based techniques [196].

Blood represents a "pool" of the metabolome reflecting endogenous metabolites [186]. The composition of the blood metabolome is affected by both intrinsic and extrinsic factors. As metabolic activation of platelets is essential for the coagulation process, serum may contain higher levels of released compounds from activated platelets and erythrocytes compared to plasma [186], which potentially could affect analysis of eicosanoids. In the present study, the isolation protocol for PBMC also generated plasma. Thus, the use of plasma for analysis of circulating free AA and eicosanoids (**paper I**) enabled the best utilisation of the blood samples. EDTA was used as an anticoagulant, which is also considered a suitable additive for LC/MS based metabolomics studies [186].

Regarding the *in vitro* study (**paper II**), differences in cell number between cell lines and different exposure conditions could potentially influence levels of secreted metabolites. However, the same number of cells were seeded in both the dose-response and time-course experiments for the two cell lines, and exposure levels were sub-toxic. Furthermore, all experiments were conducted in triplicate, with unexposed controls, and repeated three times, which highly reduces possible inter- and intra-experimental differences. Thus, the number of cells releasing circulating AA and eicosanoids to cell culture media were comparable between the two cell lines.

4.1.6 Microarray, small RNA sequencing, and data analysis

Next-generation sequencing (**paper I and III**) is becoming a popular method to study expression of small RNA (sRNA), which includes miRNA, short-interfering RNA (siRNA) and PIWI-interacting RNA (piRNA). The focus of **paper I and III** was on expression of miRNA. Other sRNA will not be discussed here.

RNA sequencing (RNA-seq) is a sensitive and quantitative method to investigate expression levels of all transcripts, and, in contrast to microarray, it is not limited to prior knowledge of a reference genome. RNA-seq relies on deep-sequencing. In short, total RNA is converted to a cDNA library of fragments with adaptors attached to one or both ends [197]. Next, each fragment is sequenced from one or both ends obtaining short *reads*. These reads are then mapped to a reference genome, reference transcript, or subjected to *de novo* assembly [197]. sRNA-seq libraries are less complex than regular RNA-seq libraries, thus sequencing is

performed with a typical range of 2-10 million reads [198]. No standard "best practise" exists for analyses of sRNA data [199].

Gene expression profiling by microarray measures the expression levels of thousands of genes in different samples of RNA. Obtaining such high-throughput data can aid in identifying differentially expressed genes following e.g. chemical exposures or alterations in diseased versus healthy tissue. Microarray, as used in **paper II and III**, is a "chip-based" approach utilising microscopic arrays of cDNA printed on glass as high-density hybridization targets. Gene expression is measured as fluorescent signals emitted from mRNA hybridizing to cognate elements on the array [200].

4.1.6.1 Data analysis

Moderated *t*-statistics were used for identifying deregulated genes from the microarray data (**paper II and III**) by using *limma* (R/Bioconductor) [201]. *Limma* applies linear modelling, thus experiments can be analysed as an integrated whole with shared information between samples [202]. When the same linear model is fitted to each gene, this may aid in borrowing strength between genes to moderate residual variance using empirical Bayes posterior variance estimator [201]. *limma* may be used for experiments with a small number of RNA samples and accommodates for unequal variance without violation of the linear modelling and the empirical Bayes framework [202]. Furthermore, *limma* is suitable for differential expression analyses for RNA-seq data [202], and could have been used in **paper I and II**. Then, sequencing counts would have been log-transformed and analysed by the same linear modelling commands as for the microarray data [202]. However, *DESeq2* (R/Bioconductor) was used for differential expression analysis of sRNA-seq data. For each miRNA, a negative binominal generalized linear model (GLM) was fitted and differential expression was tested using Wald [203]. Methods based on negative binomial models take into consideration biological variability and corrects for over-dispersion, as over-dispersion from Poisson-based analysis is associated with high rates of false positive [204]. *DESeq2* is the successor of *DESeq* [203]. An advantage of *DESeq2* is the shrinkage estimator for fold changes for differential expression analysis using rlog transformation, which takes into consideration miRNA with high or low counts [203]. While *DESeq* is reported to be more conservative than *limma*, and while the latter detects more transcripts, both *limma* and *DESeq* have been shown to identify low proportions of false detections [205]. Furthermore, both packages are reported to be good at handling few replicates [205]. As no single method is optimal under all circumstances, *limma* and *DESeq2* packages were used for microarray and sRNA data analyses, respectively.

4.1.6.2 Correcting for false discovery rate

The Benjamini-Hochberg method is commonly used to control for false discovery rate (FDR) associated with performing a large number of statistical tests [206]. Because of multiple testing, many true null-hypotheses will produce small *p*-values by chance. The consequence is type 1 errors with numerous false positives. Multiple testing adjustments can reduce the extent of false positives, meanwhile simultaneously reducing power to detect true positives [207]. Especially in analyses of microarray and RNA-seq data where hundreds or thousands of null-hypotheses are tested, this may significantly reduce the power to detect true differentially expressed transcripts. Thus, filtering may be applied in order to reduce the impact of multiple testing [207]. Applying a fold-change cut-off value compared to *p*-value can aid in identifying differentially expressed genes from microarray data, while helping to control the number of false positives [208]. The relative difference, or fold change, is a basic and widely used measure for identifying differential gene- and miRNA expression data from RNA-seq [209;210]. An estimate of the FDR can aid in the selection of an appropriate cut-off [207].

4.2 General discussion

The concern of lung cancer risk associated with urban air pollution emerged in the early 1950s [2]. In 1955, extracts of DEP were shown to be tumorigenic in mouse skin painting bioassays [211]. In inhalation studies with rats, exposure to whole exhaust caused benign and malignant tumours in the lungs [212;213], whereas filtered exhaust did not [213]. Exposure to DE/DEP were associated with several adverse health effects, and studies indicated that carcinogenicity of DE/DEP were related primarily to the particulate phase [4]. A vast number of cohort studies, studies on self-reported exposures, case-control studies, and *in vivo* and *in vitro* experimental studies focusing on the carcinogenic potential of DE/DEP have been conducted [20;214;215]. However, criticism has been raised against the potential lung overload of particles in several studies, in addition to pronounced species differences between experimental animals and humans [45]. Furthermore, uncertainties in DE/DEP exposure-response relationships in human epidemiological studies have permeated the scientific debate. IARC concluded in 1989 that limited evidence existed for carcinogenicity in humans [215]. In 2012, DE/DEP was classified as carcinogenic (Group 1) after a thorough evaluation of the literature with a special emphasis on lung cancer evidence from studies in non-metal miners and truck drivers [20].

The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD 2016) reported that occupational exposure to DE/DEP pose as one of the risk factors with the largest increase in a summary measure of exposure value (SEV) over the years 1990-2016 [216]. Concurrently,

diesel technology and diesel fuel have changed over the last decades resulting in lower emission of EC, lower levels of PAH, and other aromatics present on DEP, and lower levels of sulphur and aldehydes in the gas phase [21;22].

Few countries have established an OEL for DE/DEP or associated constituents [48]. OEL is not intended to take into consideration particularly vulnerable groups and potential susceptibility in younger or older individuals, and have a limited timeframe for the exposure scenario reflecting a workday [27]. Consequently, on- and off-road diesel vehicles are subjected to different emission regulations and higher levels of PM is permitted from off-road diesel engines [45;46].

The present project was initiated partly based on STAMI's involvement in exposure assessments of DE/DEP in Norwegian working environments and efforts related to establishing an OEL. Despite reduction efforts, workers engaged in tunnel construction in Norway are exposed to relatively high levels of DE/DEP measured as EC [41;43;44]. The process of exchanging traditional diesel powered equipment is slow due to the longevity of the existing machinery and the costs of implementing new, and less polluting, equipment. Thus, both present day and historical exposures may contribute to increased risk of adverse health effects in the years to come. Different job groups participate in tunnel construction and the group of TFW has been little studied. TFW experience high levels of DE/DEP in their working environment. Therefore, TFW constitute a well-suited group to study biological alterations related to working in a DE/DEP polluted environment.

The main topic of this thesis was to investigate the biological effects of exposure to DE/DEP, both *in vivo* in TFW and *in vitro* using immortalised HBEC in culture. In that respect, a comparative *in vitro* study was performed to explore toxic effects of CSC and B[*a*]P compounds, with focus on carcinogenesis of the lung.

In **paper I**, biological effects related to tunnel finishing work with high levels of DE/DEP in the working atmosphere were investigated. P**aper II** was designated the capability of the reference DEP (SRM2975) from a forklift engine to transform a normal HBEC line (HBEC3) *in vitro* after long-term exposure. Following the transformation study, early toxic responses to DEP were investigated in parental HBEC3 and the DEP transformed cell line T2-HBEC3. **Paper III** focused on identifying molecular alterations in the *in vitro* transformation process and aimed to clarify similarities and differences between transformed HBEC lines established after long-term exposure to DEP, the complex mixture of different carcinogens present in CSC,

or B[*a*]P being a model PAH. The chemical transformation of HBEC2 and HBEC12 by long-term exposures to CSC or B[*a*]P was previously published [193]. Figure 5 depicts a brief outline of the transformed cell lines investigated in **paper III.**

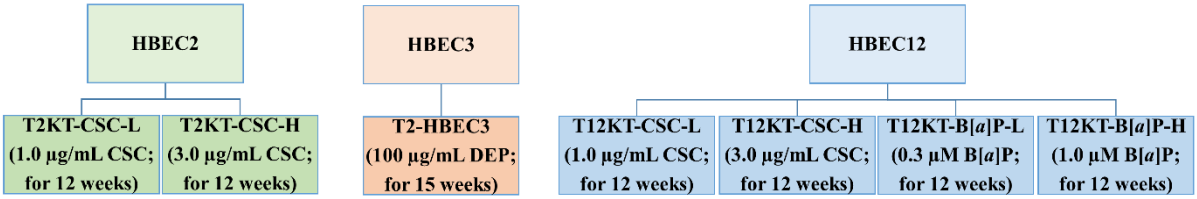

Figure 5. A schematic overview of the different chemically transformed cell lines.

4.2.1 Occupational exposure to DE/DEP during tunnel finishing work is associated with biological alterations in PBMC and plasma in tunnel finishing workers

Paper I reports results from a cross-sectional study investigating biological markers in PBMC and plasma from DE/DEP exposed TFW compared to a referent group. Several studies have investigated acute effects of exposure to DE/DEP in humans, but fewer studies have focused on chronic effects related to long-term exposures in occupational settings [184]. The inflammatory responses following these two different scenarios of acute and chronic exposures may induce distinct biological alterations [184].

PAH present on DEP may induce expression of CYP enzymes, which are important metabolising enzymes in PAH bioactivation to reactive metabolites [59]. In **paper I**, significantly higher levels of *CYP1B1* expression and DNA adducts were measured in PBMC from TFW compared to referents. Interestingly, the correlation between *CYP1B1* expression and DNA adducts was not statistically significant. Bronchial DNA adduct formation is associated with increased risk of lung cancer [82], whereas only a weak association is reported for DNA adduct levels in WBC from highly PAH exposed individuals [217]. The level of DNA adducts is highly affected by an individual's expression of biotransforming enzymes and DNA repair capacity [71;72]. Furthermore, reactive metabolites derived from nitroarenes through nitro-reduction pathways may participate in inducing DNA damage [61]. Considering inherent individual differences in DNA adduct formation and repair, individual susceptibility may be of importance evaluating the carcinogenic risk following occupational exposure to DE/DEP.

Deregulation of several KEGG pathways was identified in **paper I**, and fatty acid- and steroid biosynthesis related pathways, cell cycle, and proteoglycans in cancer may be among the most important. Recently, exposure to air pollution has been associated with increased risk of

developing type 2 diabetes [218]. Combined with the increased risk of cardiovascular diseases following occupational exposure to DE/DEP [14;15], deregulation of fatty acid biosynthesis and metabolism may potentially be involved in some of the detrimental effects. Deregulation of cell cycle progression may render cells with increased proliferative capacity [219], and altered expression of cell cycle related genes has been reported in lung cells exposed to organic components from DEP *in vitro* [220]. Deregulation of steroid biosynthesis is a target for endocrine disrupting chemicals, including PAH [69]. Moreover, cross-talk between the ER- and AHR pathways may affect AHR regulated genes [63;64], and CYP1B1 has the ability to hydroxylate E2 forming the genotoxic metabolite 4-OH-E2 [67]. Formation of 4-OH-E2 may participate in DNA adduct formation. Deregulation of steroid biosynthesis was also observed in **paper II** in both HBEC3 and T2-HBEC3 after short-term DEP exposure. Combined, results may indicate potential mechanisms for associations between DE/DEP exposure, deregulated miRNA, and DNA adducts in PBMC.

Analyses of eicosanoids and AA showed higher levels of AA and 15-HETE, and lower levels of PGD_2 and 9-HODE in plasma from TWF compared to referents. 15-HETE is associated with vascular pathogenesis and development of cancer [174], and increased levels have been reported in plasma from PM exposed mice [221]. This study also reported increased levels of PGD_2. The majority of studies indicate anti-tumour activities of PGD_2 [164]. While **paper I** reports reduced levels of 9-HODE in never-smoking TFW compared to never-smoking referents, increased levels of 9-HODE have been measured in plasma from PM exposed mice [176]. This may indicate antioxidant effects in TFW. Interestingly, plasma lipid peroxidation products have been reported as suitable biomarkers of the biologically effective dose of PM reflecting oxidative stress reactions in the airways [168]. Effects on circulating eicosanoids and AA have previously, to our knowledge, not been investigated following occupational exposure to DE/DEP. Considering the complex network of immunomodulatory effects conveyed by AA- and LA metabolism combined with the limited number of measured samples, more research is needed to elucidate the biological effects of these results.

Recent exposure measurements conducted during tunnel finishing work in Norway reported a GM EC concentration of 37.8 $\mu g/m^3$ [41]. The estimated lifetime risk of lung cancer deaths following occupational exposures to 25 $\mu g/m^3$ of EC is 689 per 10,000 [27], which exceeds acceptable levels for occupational risk. A similar estimation was conducted in an Australian study with metal and non-metal miners, where a DE/DEP exposure of 44 $\mu g/m^3$ was associated with additional 38 lung cancer deaths per 1000 [42]. Occupational exposure to DE/DEP also

increases the risk on non-cancer adverse health effects [14-16]. The results presented in **paper I** show significantly higher levels of DNA adducts in PBMC from TFW compared to referents. Combined with results obtained from sRNA-seq and circulating eicosanoids and free AA, this study may indicate potential mechanisms for adverse health effects associated with present day exposure to DE/DEP. Moreover, considering the estimated lifetime risks associated with EC exposure levels well below those recently reported during tunnel finishing work [41], combined with the marked reduced EC levels from tunnel construction over the last two decades [43;44], TFW may be at increased risk of developing adverse health effects. More studies are needed to clarify potential adverse health effects associated with tunnel finishing work and other DE/DEP exposed occupations.

4.2.2 DEP exposure transformed a human bronchial epithelial cell line in vitro rendering transformed cells with altered sensitivity to renewed exposures

The importance of DNA damaging effects of DEP/DEP constituents is well accepted [55;57], but questions as to the potential of DEP to transform normal lung cells *in vitro* remain to be answered. Furthermore, the more precise role of oxidative stress and inflammatory reactions are not fully characterised [55;57]. Although many studies have investigated toxicity of DEP extracts in different cancer cell lines or in cell lines immortalised by viral oncogenes, fewer studies have focused on the effects of whole particle exposures in immortalised HBEC lines with a normal phenotype [214]. The carcinogenic potential of DEP is debated [26], and more studies are needed to investigate the effects of not only DEP extracts, but also of whole particles including their adsorbed compounds.

One of the main findings in **paper II** is that long-term exposure to reference DEP, with low levels of organic carbon, has the capacity to stably transform an immortalised HBEC line *in vitro*, supporting carcinogenicity of DEP. However, the transformation efficacy (TE) was low (0.39%). Similar assays have previously been used to investigate the carcinogenic potential of methylnitrosurea, B[*a*]P-7,8-dihydrodiol-9,10-epoxide-1, CSC, and B[*a*]P, and various clones with different acquired traits have been established [193;194]. TE in one of these studies ranged between 0.2%-3.0% depending on the chemical exposure [194].

Several DEP transformed clones were established in **paper II**. Gene expression of EMT markers varied between the clones, but all showed downregulation of the epithelial marker *CHD1* and upregulation of mesenchymal *VIM*. EMT is known to be a plastic process, and epithelial cancer cells may retain various levels of epithelial traits [148]. Variable expression levels of EMT markers in *in vitro* transformed cells have been reported in other studies

[156;193]. Based on the relative expression of EMT marker genes, T2-HBEC3 was selected as a model clone for the further studies. Interestingly, T2-HBEC3 showed reduced migration capacity compared to parental cells and did not invade a reconstituted matrigel. Thus, it may be interpreted that T2-HBEC3 had acquired traits of EMT. Several cancer related pathways were deregulated at baseline in T2-HBEC3 when compared to parental HBEC3, and in particular the MAPK- and mTOR signalling pathways have important roles in lung cancer [222].

When assessing differences in toxic effects to renewed DEP exposure between HBEC3 and T2-HBEC3, pathways related to steroid biosynthesis and lipid synthesis were commonly deregulated. Correlations between steroid hormones and human NSCLC progression has been reported [62]. In support of these data, deregulation of the steroid biosynthesis pathway by miRNA was found in **paper I**. Pathways related to inflammation and metabolism were specifically deregulated in T2-HBEC3. Interestingly, parental HBEC3 showed higher steady-state levels of *IL1B* expression and release of IL-1β following exposure to DEP compared to T2-HBEC3. Higher levels of both *CYP1A1* and *CYP1B1* were measured in T2-HBEC3 compared with HBEC3 in the DEP short-term exposure experiments, indicating a greater xenobiotic response in transformed cells. *CYP1A1* may constitute a sensitive biomarker of DEP induced effects [223], and a significant correlation between expression of *CYP1A1* and level of DNA adducts in lung tissue has been reported [66]. Interestingly, *CYP1A1* was not expressed in PBMC from TFW (**paper I**). Nonetheless, the results presented in the present study support an inverse relationship between induction of CYP enzymes and inflammation [223;224]. A recent study conducted in BEAS-2B cells exposed to low levels of DEP for 6 months, reported increased expression of *CYP1A1* [225]. However, in contrast to the present findings (**paper II**), no effects on genotoxicity, oxidative stress, or inflammation were found. The discrepancy with our findings may be due to the use of a different reference DEP (SRM1650b [225] versus SRM2975 (**paper II**)), a marked lower exposure concentration (2 µg/mL [225] versus 100 µg/mL (**paper II**)), and a viral oncogene immortalised cell line.

Generally, the major prostaglandin species PGE_2 and $PGF_{2\alpha}$, and free AA were released in higher, unaltered levels from T2-HBEC3 compared with parental HBEC3 in the short-term exposure experiments. From HBEC3, secreted levels of PGE_2 and $PGF_{2\alpha}$ increased dose-dependently after exposure to DEP, while the levels of AA decreased. The role of PGE_2 in inflammation and cancer is well documented [164], and exposure to DEP has been shown to induce secretion of AA and PGE_2 in primary canine and rat AM [226;227]. Combined, data on

eicosanoid secretion indicate that HBEC3 elicits a significant inflammatory response to short-term DEP exposure meanwhile T2-HBEC3 show indications of constitutive sensitisation.

Combined, the results presented in **paper II** supports the carcinogenic potential of DEP. Furthermore, the results adds further knowledge on immunomodulatory effect markers following exposure to DEP and differences between normal and sensitised human bronchial epithelial cells.

4.2.3 Molecular alterations in chemically induced in vitro transformation

Tobacco smoking is the main aetiological factor for lung cancer development, but environmental and occupational exposure to air pollution, DE/DEP, and other sources of PAH compounds constitute additional risk factors [100]. Although many molecular alterations in lung cancer have been described, unknown oncogenic drivers accounts for a great number of cases [123]. More knowledge is needed on the mechanisms in lung carcinogenesis.

In P**aper III**, the aim was to further investigate the process of chemically induced *in vitro* transformation of normal HBEC lines. Long-term exposure of HBEC3 to DEP induced molecular alterations rendering cells with the potential to grow anchorage-independently in soft agar assay (**paper II**). A stable model DEP transformed cell line, T2-HBEC3, was established showing traits of EMT (**paper II**). A previous study established stably transformed cell lines following long-term exposures to different concentrations of either the chemically complex CSC or the model PAH B[*a*]P [193]. An overview of the different transformed cell lines and their parental origin is depicted in Figure 5. Although all the transformed cell lines showed traits of EMT, including mesenchymal morphology, differences in expression of EMT marker genes and invasive capacities existed between the transformed cell lines. In **paper III**, commonly and uniquely deregulated genes and miRNA and associated signalling pathways were identified in the transformed cell lines by gene expression profiling and sRNA-seq.

Principal component analysis (PCA) plots based on total gene- or miRNA expression profiles indicated fewer alterations in the T12KT transformed cell lines compared with the other transformed cell lines. These results support the less clear pattern of EMT marker genes in the T12KT transformed cell lines. Interestingly, the T2KT transformed cell lines were the only transformed cells that had gained invasive capacities and the T2KT transformed cell lines were also the cells that clustered furthest away from their parental (HBEC2) cell line. This indicates that more alterations may have occurred in these cells.

Interestingly, long-term exposures to CSC induced colony formation both in HBEC2 and HBEC12, while exposure to B[*a*]P only induced colony formation in HBEC12. When considering the inter- and intra-individual differences between clones of HBEC12 and T12KT, it may be speculated that HBEC12 may be more readily transformed than HBEC2 and HBEC3. *In vitro* transformation following long-term DEP exposure was solely performed in HBEC3 (**paper II**).

Deregulated signalling pathways based on total gene- and miRNA expression profiles for the transformed cell lines were mainly related to carcinogenesis, EMT, and the extracellular matrix (ECM). Similarities between pathway alterations and associated deregulated genes and miRNA may suggest their general importance in the intricate network of chemically induced *in vitro* transformation. However, some differences were apparent between cell lines of different chemical transformations, suggesting that multiple routes of action may participate in the transition from an epithelial to a mesenchymal cell. Moreover, it differed to some extend which transformed cell lines showed deregulation of particular pathways depending on whether the results were obtained from gene- or miRNA expression profiling.

Based on gene expression profiling, *SOX15* (a reported EMT repressor [228]) and *PTPRF* (associated with EGFR signalling pathway [229]) were the only genes commonly deregulated (↓), highlighting their general importance for *in vitro* transformation. In contrast, *LAMA3* (a component of the basement membrane [230]) was deregulated in all transformed cell lines except for T2-HBEC3. Thus, although *LAMA3* appears to be important in the transformation process, T2-HBEC3 was transformed without deregulation of this gene.

Twenty-four miRNA were commonly deregulated in the transformed cell lines. Of special importance are miR-200c-3p, miR-200c-5p, and miR-141, belonging to the miR-200 family of EMT regulators [231], which were also among the top 10 most up/downregulated miRNA in the transformed cell lines. Other *in vitro* studies are in line with the presented results [232]. As one miRNA may target several genes, the complex deregulation of multiple miRNA may participate in inducing several of the different characteristics of the transformed cell lines.

All transformed cell lines were able to form anchorage-independent colonies in soft agar, which was the trait that was used to define cell transformation. As both general and specific alterations in gene- and miRNA profiles were found, it may be speculated that there are multiple routes of action deregulating the cellular machinery during EMT. EMT is a plastic process and cancer cells of epithelial origin may harbour various intermediates between the strictly epithelial or

strictly mesenchymal states [148]. Of importance is the finding that both the epithelial marker *CDH1* and the pioneer transcription factor *FOXA2* were deregulated in all transformed cell lines. Downregulation of *CDH1* is associated with the cadherin-switch, which is a hallmark of EMT [149]. As downregulation of *FOXA2* has been reported in lung cancer cell lines and NSCLC [233;234], and is suggested to have an important role in EMT [235;236], the present finding is in support of these reports. Combined, it may be interpreted that deregulation (↓) of *CDH1* and *FOXA2* may be essential for *in vitro* lung cell transformation. Their implications for *in vivo* lung carcinogenesis needs to be further investigated.

Differences in expression of inflammatory genes was measured between the transformed cell lines, although T2-HBEC3 and the T2KT transformed cell lines generally showed similar results. Interestingly, results indicated reduced inflammatory reactions in these cells, contrasting to that inflammation is considered important in carcinogenesis [111;237].

Interestingly, expression of *CYP1B1* was increased in all the transformed cell lines, whereas expression of *CYP1A1* was reduced or unaltered. Deregulation of genes in the AHR pathway may play a role in modifying inflammatory responses in the transformed cell lines, as a complex cross-talk may exist between the AHR pathway and inflammation [238]. This was also discussed in **paper II** related to DEP short-term exposure experiments.

To conclude, **paper III** reports that both general and specific changes in gene- and miRNA expression profiles were identified in the different chemically *in vitro* transformed cell lines. Deregulated genes and miRNA and their related signalling pathways were commonly associated with carcinogenesis, EMT, and ECM. It is interesting to note that although several alterations were of general characters, there appears to be multiple routes of action during *in vitro* transformation rendering the cells with the potential to grow anchorage independently in soft agar. Further studies are, however, needed to translate the present findings to *in vivo* lung carcinogenesis.

5 CONCLUSIONS

This project reports that present day's occupational exposure to DE/DEP during tunnel finishing work is associated with biological alterations in TFW, which potentially may have implications on health outcomes by affecting carcinogenesis, lung homeostasis, inflammatory responses, and the cardiovascular system (**paper I)**. Combined, the results presented in **paper I** may be taken into consideration for general risk assessment and preventive strategies.

The project also reports that DEP has the potential to transform human bronchial epithelial cells *in vitro* (**paper II**). The stably transformed T2-HBEC3 cell line showed evidence of EMT and marked differences in baseline gene expression profiles compared with the parental cell line (HBEC3). In addition, T2-HBEC3 showed altered sensitivity to short-term DEP exposure regarding expression of genes involved in xenobiotic and lipid metabolism, and inflammation (**paper II**). Combined, the results presented in **paper II** add information of immunomodulatory effect markers following exposure to DEP, in addition to basal differences between parental and DEP transformed HBEC.

Third, the project reports general and specific changes in gene- and miRNA expression profiles identified in HBEC lines transformed *in vitro* by either DEP, CSC, or B[*a*]P (**paper III**). Deregulated genes and miRNA were mainly affecting carcinogenesis, EMT, and the ECM. Combined, **paper III** adds knowledge of both general and specific molecular events in the chemically induced *in vitro* transformation process.

Thus, although the potential of DE/DEP to cause cancer is under debate, *in vivo* and *in vitro* results obtained in this thesis are in support of DE/DEP having carcinogenic capabilities. In addition, analyses of stably transformed HBEC lines showed both general and specific changes at the gene- and miRNA level that increase the understanding of molecular alterations during the chemically induced *in vitro* transformation processes.

6 FUTURE PERSPECTIVES

More research is needed to further assess the potential adverse health effects associated with occupational exposure to DE/DEP, in particular related to tunnel finishing work. In that regard, it would be interesting to further investigate biological alterations in the sample material collected during the *in vivo* study.

Some of the data from sRNA-seq performed on samples of PBMC from TFW and referents remains to be studied. Thus, the biological effects of other small RNA such as piwi-interacting RNA can be readily analysed. Investigation of DNA methylation as a potential biomarker for DE/DEP exposure could be conducted either on genes of interest or by genome wide analyses. DNA methylation patterns could be compared with the miRNA expression profiles potentially increasing knowledge of epigenetic mechanisms associated with tunnel finishing work.

Telomere shortening can be induced by chemical exposures. As significantly more DNA adducts were measured in PMBC from TFW as compared to referents, it would be interesting to investigate if differences exist between the groups concerning telomere length in DNA from PBMC. Moreover, as alterations in plasma eicosanoids were found in TFW compared to referents, it would be interesting to analyse protein profiles in these samples. This could increase knowledge of potential effects on proteins related to inflammation and cancer. A method for investigating nitro-PAH haemoglobin adducts by high-performance liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC-APCI-MS/MS) from exposure to DE/DEP was recently published. It would be very interesting to apply this method on our sample material.

This thesis reports results obtained from Norwegian tunnel finishing workers. It would be of importance to compare the present findings in Norway with tunnel finishing work in other countries. Moreover, it could be interesting to investigate biological alterations in TFW working with biodiesel-fuelled equipment.

It is important to perform *in vitro* studies to investigate the toxic potential of the particles present in the tunnel atmosphere during finishing work. Furthermore, several interesting target genes and miRNA were identified in the *in vivo* study, and *in vitro* studies elucidating the mechanisms behind measured effects are needed. Thus, particles could be collected from the working atmosphere during tunnel finishing work to compare their toxic potential with that of the reference DEP used in the present project. Chemical characterisation of the particles would thus

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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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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/oemed-2018-105445).

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Received 21 August 2018
Revised 4 October 2018
Accepted 21 October 2018

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 ^{32}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_2 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 homoeostasis, 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.

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.

INTRODUCTION

Occupational exposure to diesel exhaust (DE) is a great health concern,[1 2] and is associated with respiratory and cardiovascular diseases (CVD).[3] DE is classified as carcinogenic to humans,[2] and this is supported by in vitro studies.[4 5] Of particular concern is the use of heavy-duty diesel engines in enclosed environments such as underground mining and tunnel construction.[1] 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 to DE from heavy-duty diesel-powered equipment.[6] 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^3 (SD=2.7 µg/m^3). GM level of NO_2 was 128.0 µg/m^3 (SD=2.9 µg/m^3).[6] 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.[3] DE particles (DEP) are composed of EC cores with various amounts of adsorbed compounds, including polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs and some metals.[3] The chemical characteristics of DEP are affected by the fuel source, engine-type, exhaust

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To cite: Rynning I, Arlt VM, Vrbova K, *et al*. *Occup Environ Med* Epub ahead of print: [please include Day Month Year]. doi:10.1136/oemed-2018-105445

after-treatment techniques, and operating conditions.[1] Exposure to DEP increases airway and systemic inflammation, which may induce oxidative stress and DNA damage.[3 5 7] Gaseous NO_2 in DE may also cause proinflammatory reactions and oxidative stress,[8] and an independent role for both acute and chronic NO_2 exposure as risk factors for CVD development has been indicated.[9]

PAHs induce cytochrome P450 monooxygenases, which are key metabolising enzymes in PAH-bioactivation to diol-epoxides, radical cations and o-quinones.[10] 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.[11] An association between DNA adduct levels in white blood cells (WBC) and risk of lung cancer in highly PAH-exposed individuals has been reported.[12] Significantly increased levels of DNA adducts were found in lymphocytes from DE-exposed bus maintenance and truck terminal workers,[13 14] and a recent study reported significantly increased levels of DNA-strand breaks in leucocytes following DE exposure.[15]

Epigenetic changes are strongly associated with environmental exposures and are mechanistically linked to adverse health effects.[16] 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.[16] Altered expression of four miRNAs in PBMC after controlled short-term exposure of human participants to DE has been reported.[17] In a study of foundry workers exposed to metal-rich particulate matter (PM), several miRNAs affecting candidate inflammatory genes were found to be deregulated.[18] 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.[7 19 20] Plasma lipid peroxidation products are suitable biomarkers of the biologically effective dose of PM and are shown to reflect oxidative stress in the airways.[21] Increased levels of 8-iso-$PGF_{2\alpha}$, 12-HETE and 13-HODE were found in plasma from DE-exposed mice.[7] A role for lipid peroxidation in mediating PM-induced atherosclerosis was reported in mice with increased plasma levels of 9-HODE and 12-HETE.[22] Additionally, proinflammatory and cancer-promoting effects were reported for PGE_2 and by contrast, PGD_2 was identified as suppressor of cell invasion and generally was reported as antitumourigenic.[19] Increased levels of 5-HETE, 12-HETE and 15-HETE are also linked to carcinogenesis and CVD.[7 20] 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 ^{32}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.

^{32}P-postlabelling

DNA was isolated from PBMC by standard phenol/chloroform extraction. The nuclease P_1 enrichment version of the thin-layer chromatography ^{32}P-postlabelling assay was used to determine bulky DNA adduct formation. The procedure was performed essentially as described,[10] 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_1 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/10^8 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 ΔΔCt 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.[4] PGI_2 was determined as its non-enzymatically hydrolysed product 6-keto-$PGF_{1\alpha}$.

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 relevant parameters were not significantly different between TFW and referents.

Table 1 Demographics

	Referents	TFW	P values
Participants	n=69 (%)	n=69 (%)	
Age			
Overall mean±SD	50.0±13.2	41.0±12.2	$6.0x10^{-5}$*
Gender (%)			
Male	69 (100)	69 (100)	
Ethnic group (%)			
Caucasian	69 (100)	69 (100)	
Smoking history (%)‡			0.003†
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)§	27.5 (3.7)	27.2 (3.9)	
Respirator mask (%)¶			0.001†
Yes, often	1 (1)	5 (7)	
Yes, rarely	26 (38)	43 (62)	
No	42 (61)	20 (29)	
Respiratory diseases (%)**††			
Chronic bronchitis	3 (4)	1 (1)	
Chronic obstructive lung disease	1 (1)	0 (0)	

*Student's t-test.
†Pearson χ^2.
‡Two cases (4%) from the referent group were missing data on smoking status.
§Eight cases (12%) from the referent group and two cases (3%) from the TFW group were missing data on BMI.
¶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.
††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.

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.

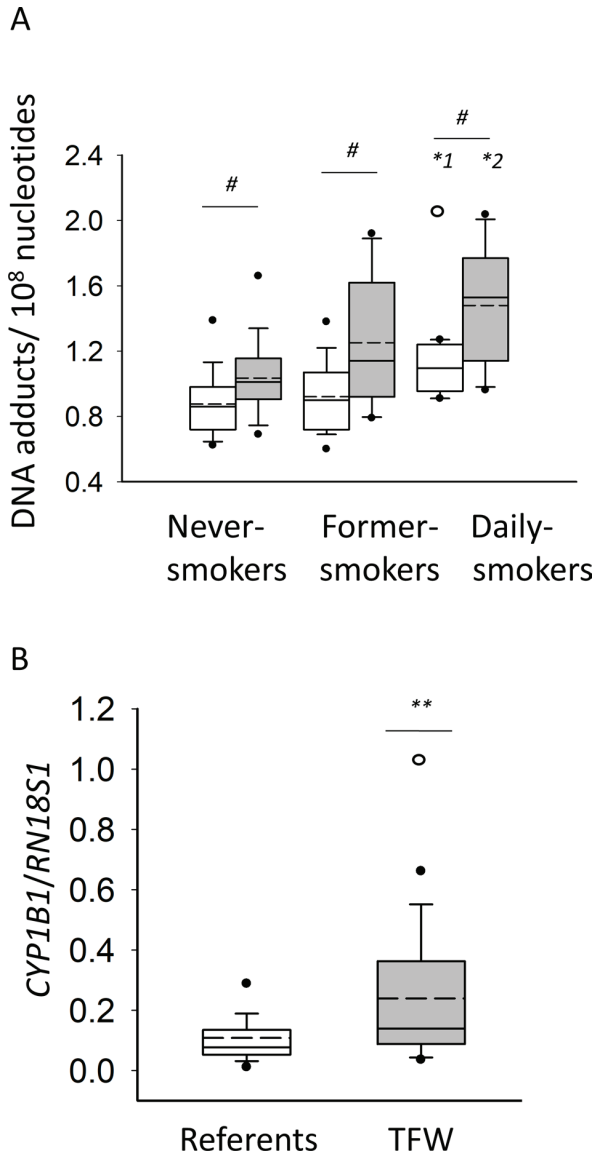

Figure 1 Bulky DNA adducts and CYP1B1 gene expression in PBMC from TFW and referents. (A) DNA adducts per 10^8 nucleotides in three smoking categories; never-smokers (n=27/17), former-smokers (n=19/16) and daily-smokers (n=7/21). (B) Gene expression of CYP1B1 in referents (n=36) and TFW (n=41). Box plots 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 (*[1]) and the TFW group (*[2]), 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 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.

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.

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_2 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	**0.002**
		Daily-smokers	24	336.6 (124.9)	298.5 (195.1, 577.3)	**<0.001**	
PGD_2 (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)	**0.034**	**0.002**
		Daily-smokers	24	14.9 (8.3)	13.9 (4.0, 27.9)	**0.004**	
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	**0.041**
		Daily-smokers	23	48.9 (23.3)	44.8 (17.1, 83.4)	**0.005**	
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)	**0.033**	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<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_2, prostaglandin D_2; TFW, tunnel finishing worker.

DISCUSSION

Detailed exposure measurements of dust, EC, organic carbon, and NO_2 have been reported from various job groups related to drilling and blasting during tunnel construction in Norway.[23] However, DE/DEP exposure from heavy-duty diesel-powered equipment during tunnel finishing work was only recently investigated.[6] 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.[11] 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,[13 14 24] and positive correlations between cigarette smoking and DNA adducts in PBMC.[11] Taking into account that DNA adducts in bronchial tissue may predict lung cancer risk,[25] and that adducts in PBMC can be considered a distant surrogate of pulmonary exposure,[11] 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.[26] AHR and ARNT were equally expressed in both groups. A crosstalk between the oestrogen receptor (ER) and the AHR pathway exists.[27] Several PAHs present in DEP are shown to directly activate ERα,[27] indicating that CYP1B1 may be induced by non-AHR-mediated mechanisms. Alternatively, endogenous availability of coactivators may play a role.[27] 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.[16] 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.[28] miR-31–5 p 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.[16] In agreement with the present study, downregulation of miR-652–3 p was also reported in human blood after short-term PM_{10} exposure. This miRNA has a putative role in CVD.[29]

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),[30–32] while others may have roles in Alzheimer's disease, chronic heart failure, and cell migration and invasion (miR-6513–3 p and miR-660–3 p).[16 33] Both miR-20b-5p and miR-196b-5p may be important regulator of apoptosis and tumourigenesis.[32] 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.[34] Subchronic exposure to low concentrations

of DE is shown to deregulate genes related to fatty acid biosynthesis in mice.[35] miRNAs may regulate cell cycle progression,[36] and altered expression of cell cycle-related genes has been reported after exposure to organic components from DEP.[37] Steroid biosynthesis is a target for endocrine disrupting chemicals, including PAHs.[27] Interestingly, human bronchial epithelial cells exposed to DEP in vitro also showed deregulation of this pathway.[4] Deregulation of proteoglycans in cancer may be of importance as this pathway of extracellular matrix constituents is emerging as highly affecting homoeostasis and progression of various diseases, including cancer.[38] 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 homoeostasis, 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.[39] 15-HETE signalling is associated with cardiovascular pathogenesis, as well as cancer development.[20] While we report reduced levels of PGD_2 in TFW, an increase in this metabolite was measured in plasma from PM-exposed mice.[39] Interestingly, the great majority of studies indicate antitumourigenic activities of PGD_2.[19] 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,[22] but the modulation of oxidative stress and antioxidant defence processes may be time and dose dependent. Evidence indicates that gaseous NO_2 is an independent risk factor for CVD.[9] Since NO_2, together with DEP and other DE constituents, can induce oxidative stress and inflammation,[3 5 7 8] it may be speculated that NO_2 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_2 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.[40] 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.[4 5]

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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Acknowledgements We thank Rita Bæra for skillful technical assistance. We also thank the workers and the tunnel construction companies participating in this study, in particular Kristin H Hovland (Occupational Health Physician).

Contributors Study design was led by IR and SM, with contributions from BU, PR, VMA, DHP, MM, JT and AH. IR, EP and BU carried out enrolment of participants and collection of human samples. Data collection was carried out by IR, VMA, KV, JN and PR. IR, ØS and JK conducted statistical analyses. IR drafted the manuscript. All authors participated in data analysis, critically revised the manuscript and approved the final version.

Funding This work was financially supported by the Statoil Working Environment Fund (Norway) and the Ministry of Youth, Education and Sports of the Czech Republic (LO1508 to PR, JK and KV, LM2015073; CZ.02.1.01/0.0/0.0/16_013/0001821 to JT; the grant Research Center for Informatics CZ.02.1.01/0.0/0.0/16_019/0000765 to JK; project "FIT", no. CZ.02.1.01/0.0/0.0/15_003/0000495 to MM and JN). Work at King's College London was supported by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Health Impact of Environmental Hazards at King's College London in partnership with Public Health England and Imperial College London.

Disclaimer The views expressed in this article are those of the authors and not necessarily those of the UK National Health Service, the UK National Institute for Health Research Health, the UK Department of Health and Social Care or Public Heath England.

Competing interests None declared.

Patient consent Not required.

Ethics approval The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (REC #2014/2199).

Provenance and peer review Not commissioned; externally peer reviewed.

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TOXICOLOGICAL SCIENCES, 166(1), 2018, 51–64

doi: 10.1093/toxsci/kfy183
Advance Access Publication Date: July 16, 2018
Research Article

In Vitro Transformation of Human Bronchial Epithelial Cells by Diesel Exhaust Particles: Gene Expression Profiling and Early Toxic Responses

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ABSTRACT

Occupational exposure to diesel exhaust may cause lung cancer in humans. Mechanisms include DNA-damage and inflammatory responses. Here, the potential of NIST SRM2975 diesel exhaust particles (DEP) to transform human bronchial epithelial cells (HBEC3) *in vitro* was investigated. Long-term exposure of HBEC3 to DEP led to increased colony growth in soft agar. Several DEP-transformed cell lines were established and based on the expression of epithelial-to-mesenchymal-transition (EMT) marker genes, one of them (T2-HBEC3) was further characterized. T2-HBEC3 showed a mesenchymal/fibroblast-like morphology, reduced expression of *CDH1*, and induction of *CDH2* and *VIM*. T2-HBEC3 had reduced migration potential compared with HBEC3 and little invasion capacity. Gene expression profiling showed baseline differences between HBEC3 and T2-HBEC3 linked to lung carcinogenesis. Next, to assess differences in sensitivity to DEP between parental HBEC3 and T2-HBEC3, gene expression profiling was carried out after DEP short-term exposure. Results revealed changes in genes involved in metabolism of xenobiotics and lipids, as well as inflammation. HBEC3 displayed a higher steady state of *IL1B* gene expression and release of IL-1β compared with T2-HBEC3. HBEC3 and T2-HBEC3 showed similar susceptibility towards DEP-induced genotoxic effects. Liquid-chromatography-tandem-mass-spectrometry was used to measure secretion of eicosanoids. Generally, major prostaglandin species were released in higher concentrations from T2-HBEC3 than from HBEC3 and several analytes were altered after DEP-exposure. In conclusion, long-term exposure to DEP-transformed human bronchial epithelial cells *in vitro*. Differences between HBEC3 and T2-HBEC3 regarding baseline levels and DEP-induced changes of particularly *CYP1A1*, IL-1β, PGE_2, and $PGF_{2\alpha}$ may have implications for acute inflammation and carcinogenesis.

Key words: diesel exhaust particles; human bronchial epithelial cells; *in vitro* transformation; epithelial-to-mesenchymal transition; gene expression profiling; eicosanoid secretion.

The impact of particulate air pollution on human health is of major concern worldwide (Cohen *et al.*, 2005). Exposure to particulate matter from diesel engine exhaust (DEP) is a potential health hazard, especially in larger cities with extensive traffic pollution and in occupational settings where heavy-duty diesel engines are operated in enclosed areas (Benbrahim-Tallaa *et al.*, 2012). A link between exposure to diesel exhaust (DE) and increased risk of developing respiratory and cardiovascular diseases has been established (Pope *et al.*, 2002; Sydbom *et al.*, 2001). Based on studies in miners, exposure to DE has been classified as carcinogenic to humans (Benbrahim-Tallaa *et al.*, 2012).

The particulate phase of DE consists both of fine (<2.5 μm) and ultrafine (<0.1 μm) particles, that deposit mainly in the peripheral parts of the lung where they directly may affect alveolar macrophages (AMs) and epithelial cells (Øvrevik *et al.*, 2015). Various amounts of trace metals, polycyclic aromatic hydrocarbons (PAHs) and nitroarenes may adsorb to the surface of DEP. Characteristics of DEP are affected by fuel source, engine-type and operating conditions, and their chemical variability has implications for toxicological outcomes (Øvrevik *et al.*, 2015; Westerholm and Egebäck, 1994). Although the importance of DNA-damaging effects of DEP in lung carcinogenesis are well-accepted, the more precise role of oxidative stress and inflammatory reactions are not fully characterized (Cassee *et al.*, 2013; Øvrevik *et al.*, 2015). Many studies have addressed toxicity of DEP/DEP-extracts in different cell lines, but studies concerning effects of whole particles in normal human bronchial epithelial cells (HBECs) are less frequent (Schwarze *et al.*, 2013).

Airway epithelial cells have been shown to be important in mediating innate immune responses and inflammatory signaling (Bals and Hiemstra, 2004). Sustained inflammation and cellular redox imbalance may cause genomic instability leading to abnormal cells that are prone to malignant transformation. An important event in transforming epithelial cells to cancer cells is epithelial-to-mesenchymal transition (EMT), which is a reversible process also involved in tissue-repair and development of fibrosis (Kalluri and Weinberg, 2009; Lamouille *et al.*, 2014). Several genetic and epigenetic alterations may participate in the so-called cadherin-switch, with downregulation of E-cadherin and upregulation of N-cadherin in addition to upregulation of the mesenchymal cytoskeleton marker vimentin (Lamouille *et al.*, 2014; Liu *et al.*, 2015). The transcription factors SNAI1/2, ZEB1/2, and TWIST1 all participate in repression of E-cadherin, whereas TWIST1 may also be involved in induction of N-cadherin (Pallier *et al.*, 2012; Peinado *et al.*, 2007).

In vitro cell transformation due to DEP/DEP-constituents has been little studied in HBECs with a normal phenotype. Primary rat tracheal epithelial cells were transformed *in vitro* after exposure to SRM1650-extracts, but the cells did not gain immortalization (Ensell *et al.*, 1998). Another study showed that BALB/c-3T3 cells treated with DEP from a light-duty engine formed foci of morphologically transformed cells that were tumorigenic in nude mice (Hasegawa *et al.*, 1988). HBECs immortalized with *hTERT* and *CDK4* have been suggested to represent particularly relevant models for *in vitro* lung carcinogenesis (Delgado *et al.*, 2011; Ramirez *et al.*, 2004), compared with cell lines of either cancer origin or viral oncogene immortalization (ie, A549 and BEAS-2B) (Gazdar *et al.*, 2010). Two recent studies established stably transformed HBECs after long-term exposure to benzo[*a*]-pyrene (B[*a*]P) or cigarette smoke condensate (CSC) (Bersaas *et al.*, 2016; Vaz *et al.*, 2017). Transformation of HBECs following exposure to methylnitrosourea or B[*a*]P-diol-epoxide-1, either alone or in combination, has also been reported (Damiani *et al.*, 2008). B[*a*]P and chemical species in CSC are also present in various concentrations in DEP; however, the transformation potential of intact DEP in HBECs has, to our knowledge, not been investigated.

Enzymatic and nonenzymatic oxidation of arachidonic acid (AA) yields eicosanoids with implications for inflammation and carcinogenesis. Lipid mediators, including prostaglandins (PGs), are important signaling molecules with different and sometimes opposing functions, depending on tissue and body homeostasis, as well as environmental influences (Menter and Dubois, 2012). The implication of PGE_2 in inflammation and cancer is well documented (Menter and Dubois, 2012). $PGF_{2\alpha}$, the oxidation product of both PGE_2 and PGH_2, is also an important mediator of inflammatory responses. In contrast, isoprostanes like 8-iso-$PGF_{2\alpha}$ from nonenzymatic lipid peroxidation of AA, are markers of oxidative stress (Morrow *et al.*, 1990). Primary canine AMs exposed to 100 μg DEP/ml (SRM1650a) were shown to induce formation of AA, PGE_2, and 8-iso-$PGF_{2\alpha}$ (Beck-Speier *et al.*, 2005). In a study of primary rat AM exposed to low concentrations (1 and 10 μg/ml) of low sulfur DEP, release of PGE_2 was induced, whereas higher concentrations (100 and 500 μg/ml) attenuated the responses (Bhavaraju *et al.*, 2014). Consequently, altered secretion of eicosanoids could potentially serve as mediators of DEP-exposure. However, little is known about release of eicosanoids from HBECs after exposure to DEP.

Here, the potential of the chemically well characterized DEP reference material NIST SRM2975 to transform immortalized HBEC3 *in vitro* was studied. Several DEP-transformed clones were established and characterized for EMT-markers, and 1 clone (T2-HBEC3) was subjected to further studies. Baseline differences in gene expression profiles between parental HBEC3 and T2-HBEC3 were analyzed. Next, to assess differences in sensitivity to DEP, HBEC3 and T2-HBEC3 were characterized after short-term exposure by gene expression profiling, analysis of DNA strand breaks, as well as cellular release of Interleukin-1 beta (IL-1β) and eicosanoids.

MATERIALS AND METHODS

Sonication of the DEP material. Diesel particulate matter SRM2975 (collected from the exhaust of an industrial forklift) was purchased from NIST (National Institute of Standards and Technology, Gaithersburg, Maryland). Particles were weighed and resuspended in dH_2O to a concentration of 10 mg DEP/ml. This suspension was sonicated for 15 min at 4°C with amplitude 100% and cycle 0.5 (Hielscher Ultrasound Technology, Teltow, Germany) before aliqotation and freezing at −20°C. Before suspension in exposure media, DEP aliquots were thawed and sonicated 1 min at 4°C, peak power 75.0, duty factor 5.0 and cycles/burst 200 (Covaris M220 Focused-Ultrasonicator, Brighton, United Kingdom).

Nanoparticle tracking analysis. The mean hydrodynamic size of the suspended particles (DEP mode) was determined via

nanoparticle tracking analysis (NTA) using a NanoSight NS300 instrument (Malvern Instruments Ltd, Skallestad, Norway). DEP (100 μg/ml) was prepared and sonicated as indicated above and incubated in triplicates at 37°C. After 0, 24, and 72 h 2 μl aliquots were taken from each replicate and diluted in cell culture media (1:750). This dilution was used for NTA. Per measurement, 6 movies of 30 s each were recorded. All data were analyzed with the NanoSight NTA 3.1 software (Malvern Instruments Ltd, Skallestad, Norway). The results shown are from 3 independent measurements in triplicate.

Cell culture. The *hTERT* and *CDK4* immortalized normal HBEC line HBEC3 was kindly provided by Dr John D. Minna (Ramirez *et al.*, 2004). HBEC3 was recently authenticated by the Leibniz-Institut DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). HBEC3 were maintained in a mixture (1:1) of LHC-9 (Thermo Fisher Scientific, Oslo, Norway) and RPMI 1640 (Sigma-Aldrich, Oslo, Norway) medium with 5% fetal bovine serum (Sigma-Aldrich), and plated on collagen coated (Nutragen, CellSystems, Troisdorf, Germany) dishes (Sarstedt, Oslo, Norway). Cells were maintained at 37°C in humidified atmosphere with 5% CO_2.

High-resolution field emission scanning electron microscopy. HBEC3 cells were seeded at 2600 cells/cm^2 onto collagen coated 24 mm Costar Transwell Permeable Support 0.4 μm Polycarbonate Membrane (Thermo Fisher Scientific) and placed in the incubator for 24 h. Two wells without cells were included as controls. Cells were exposed to 0 and 100 μg DEP/ml for 72 h whereas wells without cells were exposed to 100 μg DEP/ml only. After 72 h, the exposure media were discarded and the wells were washed twice with PBS before fixation in 10% neutral buffered formalin-PBS-solution (Sigma-Aldrich). Samples were then dehydrated using gradients of ethanol (EtOH) (Kemethyl, Kolbotn, Norway followed by a chemical drying series with Hexamethyldisilazane (Sigma-Aldrich) and EtOH.

The filter specimens were cut from the exposure wells and mounted on a 25-mm diameter aluminum pin stub (Agar Scientific Ltd., Stansted Essex, United Kingdom). Samples were air-dried under sterile conditions for approximately 1 h at RT before being mounted on specimen mounting stubs of 25 mm (Agar Scientific Ltd.). Double-sided carbon adhesive discs (Agar Scientific Ltd.) were used for mounting the specimens onto the stubs. The stubs were then sputter coated with 5- to 6-nm thick layer of platinum in a Cressington 208HR sputter coater (Cressington Scientific Instruments Ltd., Watford, United Kingdom). Samples were analyzed using a SU 6600 Field emission scanning electron microscopy (FESEM) (Hitachi, Ibaraki-ken, Japan) in the secondary electron imaging mode. The microscope was operated at an acceleration voltage of 10.0 kV, an extraction voltage of 1.8 kV, and a working distance of 7.4 and 7.7 mm.

DEP cell transformation assay. HBEC3 were seeded at 1.0×10^4 cells/well in quadruplicates in 6-well plates (Nunc, Thermo Fisher Scientific) and exposed to the nontoxic concentration of 100 μg DEP/ml (15.6 μg/cm^2) for 72 h followed by 72 h with media only. Control cells were exposed to the same cell culture medium without DEP. Media were changed after 72 h. The 4 technical replicates for both control and exposed cells were treated independently throughout the assay. DEP-exposed and control cells were trypsinized, reseeded at 10 000 cells/well and treated as mentioned above for the next 15 weeks. After 15 weeks, cells were seeded in 0.35% soft agar (Difco agar noble, Sigma-Aldrich). After approximately 2 weeks, colonies $\geq$ 20 μm in diameter were counted. Colonies were isolated using a micropipette and transferred to a 24-well plate where monolayers of cells were established. The soft agar assay was carried out twice to ensure true clonality and continued potential of the isolated cell lines to grow anchorage-independently. Several cell lines were established. Based on the expression of EMT-marker genes, one of the cell lines, T2-HBEC3, was selected as a model for further studies.

Cell migration and invasion analysis. Cell migration and invasion was studied using an IncuCyte Zoom Live Cell Imaging microscope and software (Essen BioScience, Mölndal, Sweden). Cells were seeded at a concentration of 6.0×10^4 cells/well in an ImageLock 96-well plate coated with matrigel (100 μg/ml, Corning Matrigel, VWR International, Oslo, Norway). A wound was made in the cell layer by the WoundMaker tool (Essen BioSciences) after 16 h. To study migration, cells were placed in the incubator containing the IncuCyte Zoom microscope and images were acquired every hour for 72 h. To study invasion capacity, the cell layer was embedded in 8 mg/ml matrigel before the plate was placed in the incubator.

Immunoblotting analysis. Whole cell extracts of HBEC3 and T2-HBEC3 were prepared and protein concentrations were measured using the BCA Assay (Thermo Fisher). Protein samples (25 μg) were run on 10% Mini-Protean TGX Stain-Free gel (BioRad, Oslo, Norway) and transferred to a PVDF membrane (BioRad). Antibodies against vimentin (V6630, Sigma-Aldrich), β-actin (MA5-11869, Thermo Fisher Scientific) and E-cadherin (EP700Y, Abcam, Cambride, United Kingdom) were used. Secondary antibodies were horseradish peroxidase-conjugated antirabbit/antimouse IgG antibodies (Cell Signaling Technology, Leiden, The Netherlands). Immunoreactive bands were detected using chemiluminescent substrate (SuperSignal West Pico, Thermo Fisher Scientific).

Short-term exposure to DEP. To assess possible differences in responses to DEP-exposure in HBEC3 and T2-HBEC3, 2 short-term exposure experiments were conducted: (1) a dose-response experiment with exposure concentrations ranging from 0 to 400 μg DEP/ml for 48 h and 2) a time-course experiment where cells were exposed to 200 μg DEP/ml for 24, 48 and 72 h, respectively. Following these exposures, several endpoints were analyzed, including gene expression profiling, analysis of DNA strand breaks, and cellular release of IL-1β and eicosanoids.

HBEC3 and T2-HBEC3 were seeded in triplicates into collagen coated 6-well plates (Nunc) at 2.0×10^5 cells/ml and left in the incubator for 24 h. Cells were then exposed either to different concentrations of DEP (0, 25, 50, 100, 200, and 400 μg/ml) (corresponding to 3.9, 7.8, 15.6, 31.3, and 62.5 μg/cm^2) for 48 h ("dose-response") or to 200 μg DEP/ml (31.3 μg/cm^2) for 24, 48, and 72 h ("time-course"). Exposure media were collected at the end of the exposures, centrifuged at 4°C, 12 000 rpm for 10 min to discard particles and stored at −20°C until further analyses. Cells on the 6-well plates were washed ×3 in ice-cold PBS and stored at −80°C for isolation of RNA.

Measurement of gene expression by RT-qPCR. Total RNA was isolated from exposed cells with Isol-RNA Lysis Reagent (5 Prime, VWR International, Oslo, Norway) and dissolved in nuclease free water. RNA quantity and quality were measured with Nanodrop 8000 (Thermo Fisher Scientific) and integrity of the

isolated RNA was ascertained on an Agilent Bioanalyzer by the use of RNA 6000 Nano kit (Agilent Technologies, Oslo, Norway). RIN values were in all cases ≥ 9. One μg of total RNA was used as input for reverse transcription with qScript cDNA synthesis kit (Quanta Biosciences, VWR International, Oslo, Norway). qPCR reactions were set up with PerfeCTa SYBR green fast mix (lo/high ROX) (Quanta Biosciences, VWR International) and run on StepOnePlus (Applied Biosystems, Thermo Fisher Scientific) or Quant Studio 5 (Applied Biosystems, Thermo Fisher Scientific). Relative gene expression was normalized to the expression of *β-actin* and calculated using the ΔΔCt method. Primers were purchased from Sigma-Aldrich or Thermo Fisher Scientific. Primer sequences used in this study are summarized in the Supplementary Table 1.

Gene expression profiling. Biotinylated complementary RNA (cRNA) was generated from 500 ng of total RNA (RIN ≥ 9) using the TargetAmp-Nano Labeling Kit for Illumina Expression BeadChip (Epicentre, an Illumina company, Madison, WI). Biotinylated cRNA targets (900 ng) was hybridized to the Illumina Human-HT12 v4 Expression BeadChips for 17 h at 58°C. Hybridization, as well as the subsequent washing, staining, and drying of the beadchips were performed according to the standard Illumina protocol. The hybridized beadchips were scanned using the Illumina iScan System and bead level data were summarized by Illumina GenomeStudio Software v2011.1 (Illumina Inc., GeneTiCA, Prague, Czech Republic). Normalized and raw bead level data are deposited in GEO with accession number GSE107481.

Measurement of cytokine release by ELISA. IL-1β release to cell culture media from the DEP dose-response experiments was measured by ELISA using the Human IL-1β/IL-1F DuoSet kit (R&D system Europe, Abingdon, United Kingdom) according to the manufacturer's instructions. Absorbance was measured and quantified using a TECAN sunrise plate reader with associated software (Magellan V 1.10, Phoenix Research Product, Hayward, California).

Comet assay. Cells were exposed to different concentrations of DEP (0, 25, 50 and 100 μg/ml) for 24 h. Cells were trypsinized and resuspended (1×10^6 cells/ml) in PBS (10 mM EDTA, without Ca^{2+}/Mg^{2+}, pH 7.5) before resuspension in 0.75% soft agar solution. In total 7 μl of this suspension was loaded in triplicates onto hydrophilic polyester films (GelBond, Lonza Rockland Inc., Maine) and lysed over night at 4°C. For analysis of oxidative DNA-damage, films were first treated for 1 h at 4°C in enzyme buffer (40 mM HEPES with 0.1 M KCl and 0.5 mM Na_2-EDTA, pH 7.6) and then for 1 h at 37°C with/without 0.5 μg/ml formamidopyrimidine-DNA-glycosylase (FPG) in enzyme buffer containing 0.2 mg/ml BSA. The FPG enzyme (crude FPG extract) was prepared with modifications as described in Olsen *et al.* (2003). DNA was unwinded by immersing the films in cold electrophoresis solution (0.3 M NaOH, 0.001 M Na_2-EDTA, pH > 13) for 40 min and electrophoresis was run at 10°C with 0.8 V/cm for 25 min with circulation as described previously in Gutzkow *et al.* (2013). After fixation in 96% EtOH, DNA was stained with SYBR GoldNucleic Acid Gel Stain (Life Technologies, Paisley, United Kingdom) diluted 1:10 000 in TE-buffer (1 mM Na_2-EDTA, 10 mM Tris-HCl, pH 8) before examination at ×20 magnification under Olympus BX51 microscope (light source: Olympus BH2-RFL-T3, Olympus Optical Co., Ltd.; camera: A312f-VIS, BASLER, Ahrensburg, Germany). Approximately 30 comets per gel were randomly counted (Comet Assay IV, Perceptive Instruments, Suffolk, United Kingdom) and DNA-damage was quantified as tail intensity (% tail DNA).

Liquid-chromatography-tandem-mass-spectrometry analysis of eicosanoids. Standards of PGE_2, PGD_2, PGA_2, PGI_2, PGJ_2, $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, 8-iso-PGE_2, 15-keto-PGE_2, 13, 14-dihydro-15-keto-PGE_2, 13, 14-dihydro-15-keto-PGD_2, and lipoxin A4 (LXA4) were purchased from Cayman Chemical Company (Michigan). Hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acid, formic acid puriss p.a. for mass spectroscopy, ethyl acetate p.a. ACS, methanol p.a. ACS and acetonitrile liquid-chromatography-tandem-mass-spectrometry (LC-MS) grade were purchased from Sigma-Aldrich (Prague, Czech Republic). Ultrapure water was obtained from a Milli-Q UF Plus water system (Millipore, Molsheim, France).

Solid-phase-extraction was used for extraction of AA metabolites from cell culture media. SELECT HLB SPE 1 ml (30 mg) cartridges (Supelco, Prague, Czech Republic) were washed with 1 ml of ethyl acetate, 1 ml of methanol and 1 ml of water. Then 1 ml of cell culture medium was loaded onto each SPE column. The columns were washed with 2 ml of water. The cartridges were air dried for 3 min with vacuum and analytes were eluted with 1.5 ml of methanol. Samples were dried under a stream of nitrogen, re-dissolved in 60 μl of methanol and aliquots of 5 μl were injected into the HPLC column.

Sample analyses were performed using LC/MS/MS. An Agilent 1200 chromatographic system (Agilent Technologies, Waldbronn, Germany), consisting of binary pump, vacuum degasser, auto sampler and thermostatted column compartment, was used. Separation of PGs was carried out using an Ascentis Express C18, 2.1 × 150 mm, 2.7 μm particle size column (Supelco, Bellefonte, Pennsylvania) with a 25-min linear gradient from 30% to 100% of acetonitrile. Mobile phase contained 0.1% of formic acid. The flow rate of the mobile phase was 0.3 ml/min, the column temperature was set at 45°C. A triple quadrupole mass spectrometer Agilent 6410 Triple Quad LC/MS (Agilent Technologies, Santa Clara) with an electrospray interface (ESI) was used for detection of the analytes. The mass spectrometer was operated in the negative ion mode. Selected ion monitoring at m/z 303.2 was used for quantification of AA and multiple reaction monitoring for other analytes.

Statistical analysis. Data output was analyzed in Sigma Plot 12.0, RStudio (2009) or StataSE 14. Mann-Whitney *U* test was used when analyzing soft agar colony formation. Multiple comparisons of DEP-transformed clones were conducted with ANOVA on log-transformed data with Dunnett's post hoc test. The dose-response experiments were analyzed by a linear mixed model (nlme package in R), with a random intercept for combinations of experiment, dose, and cell line. The residual variance was allowed to vary between cell lines. Data from time-course exposure experiments were analyzed by a linear mixed model (Stata), with random intercept for experiment. Data from migration patterns were analyzed by a non-linear mixed effects model using the nlme package (R). We considered here the following model $D\left[1 - \exp\left(-B \times \text{hour} - C \times \text{hour}^2\right)\right]$ where B indicates the cells migration rate, and D indicates the final asymptotic cell density in the wound. The C coefficient, a constant value, was added to give a closer fit between model predictions and data. Both B and D had cell line as fixed effect. In addition, nested random intercepts for cell line, experiment and well were added for B, allowing the migration rate to vary between cell lines, experiments and wells.

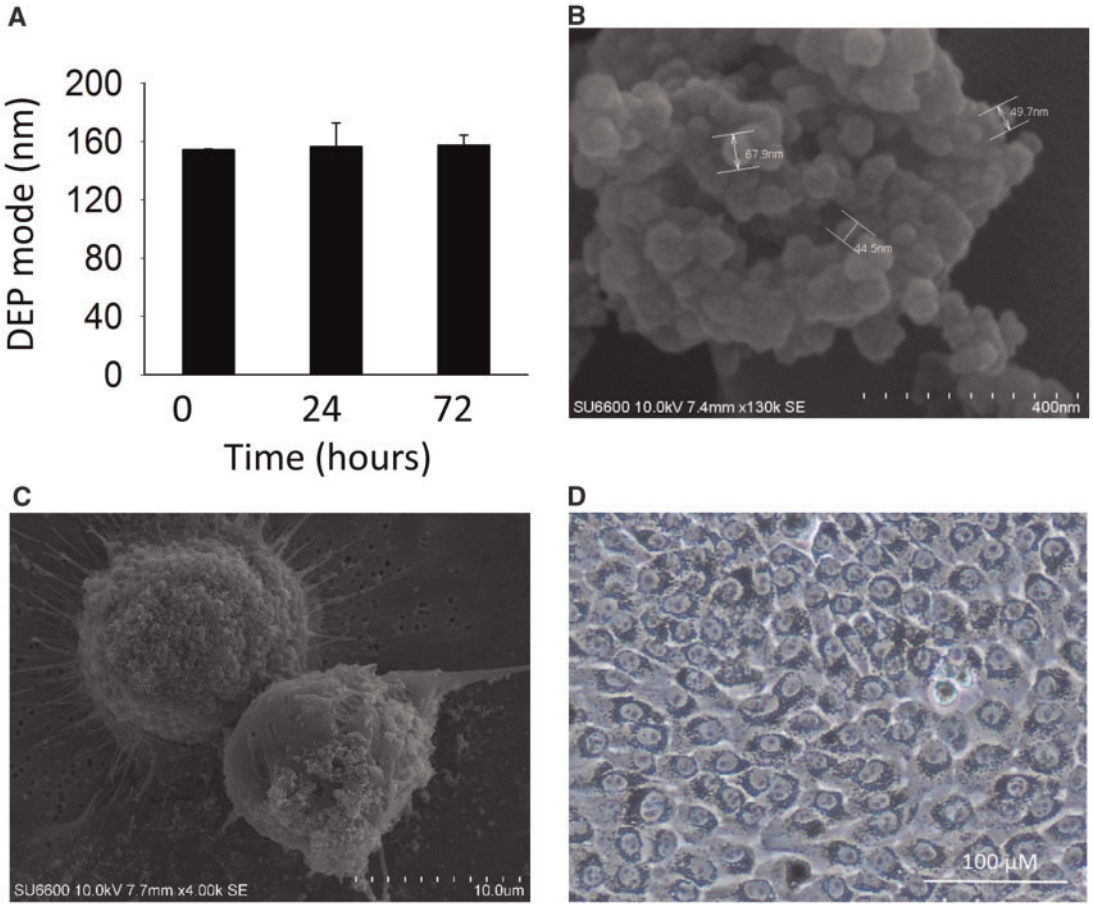


Figure 1. Exposure of cells to DEP. A, Mean hydrodynamic size of SRM2975 (DEP) in cell culture medium incubated for 0–72 h after ultrasonication. Data display mean ± SD, $n = 3$. FESEM images of DEP dispersed in cell culture medium (B) and HBEC3 exposed to 100 µg DEP/ml for 72 h (C). D, Light microscopy image (×10 magnification) of HBEC3 exposed to 100 µg DEP/ml for 48 h.

Bead summary data from microarray analysis were imported into the R statistical environment (http://www.r-project.org; last accessed April 2017) and normalized using the quantile method in the Lumi package (Du *et al.*, 2008). Only probes with a detection p-value $< .01$ in >50% of arrays were included for further analyses. Differential gene expression was analyzed in the Limma package using the moderated t-statistic. A linear model was fitted for each gene given a series of arrays using lmFit function. Multiple testing correction was performed using the Benjamini and Hochberg method. ToppFun tool was utilized for annotated genes. Goeman's global test and the KEGG database (http://www.genome.jp/kegg; last accessed April 2017) were applied to identify deregulated biological pathways and deregulated genes within these pathways. The procedure of Holm for control of the family wise error rate was applied.

RESULTS

Exposure of HBEC3 to SRM2975

The mean hydrodynamic size of ultrasonicated DEP dispersed in cell culture medium was approximately 150 nm in diameter and did not change with time (0–72 h) (Figure 1A). FESEM images were acquired for DEP dispersed in cell culture medium showing core particles with a diameter of approximately 50 nm (Figure 1B), and HBEC3 cells exposed to 100 µg DEP/ml for 72 h showed DEP attached to the cell surface (Figure 1C). Further examination by light microscopy of HBEC3 exposed to 100 µg DEP/ml for 48 h after washing indicated black staining from the particles also colocalizing with cell cytoplasm. There was a lack of staining above nuclei and at the outer edge of the cell membranes, suggesting intracellularly localization of DEP (Figure 1D).

HBEC3 In Vitro Transformation Assay

HBEC3 was exposed to the subtoxic concentration of 100 µg DEP/ml for 15 weeks before seeding in soft agar. After 14 days in soft agar, a significant increase in colony growth from DEP-exposed cells compared with controls was observed (Figure 2A). The transformation efficacy (TE) was 0.39%. Single colonies were picked from the soft agar and transferred to monolayer culture. To ensure continued potential to grow anchorage-independently in soft agar and to verify clonal origin, transformed cells were subjected to a second round of selection in soft agar. Four DEP-transformed clones were subsequently established as cell lines in monolayer culture and subjected to further investigation of expression of EMT-marker genes.

DEP-Transformed Clones Express Markers of EMT

Expression of several genes known to be involved in EMT was analyzed in 4 DEP-transformed clones. All clones showed significantly reduced expression of *CDH1* compared with the parental cell line HBEC3, while the levels varied between the individual clones (Figure 2B). Three of the clones showed upregulation of *CDH2* expression, whereas the level of expression from clone

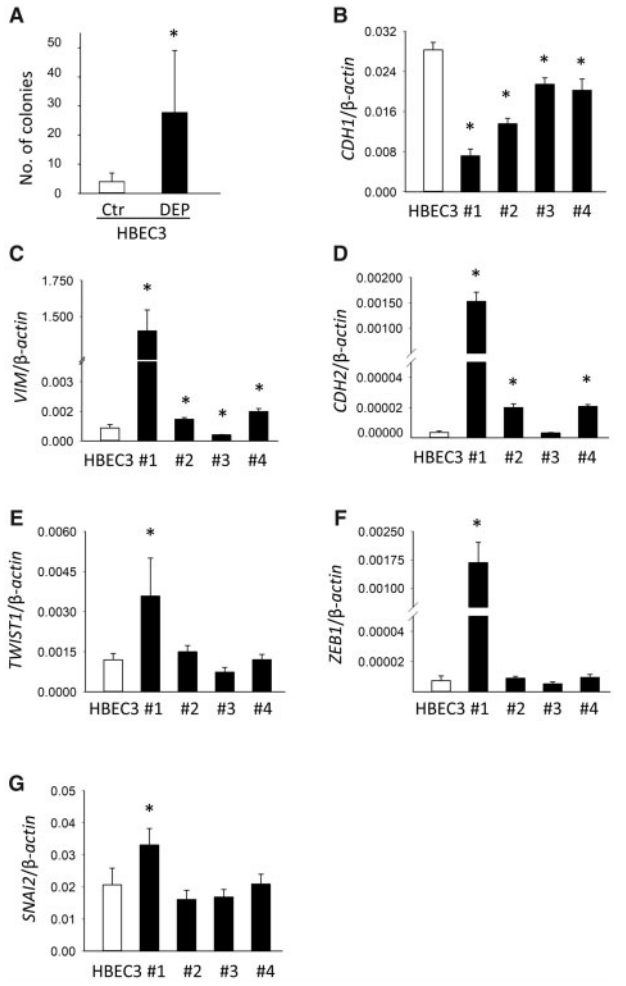


Figure 2. DEP-induced *in vitro* transformation and expression of EMT-marker genes. A, Colony formation in HBEC3 exposed to 100 μg DEP/mL for 15 weeks compared with unexposed control cells. Data display mean ± SD, $n = 4$. * $p < .05$ (Mann-Whitney *U* test). EMT-marker genes were measured in HBEC3 and DEP-transformed clones Nos. 1–4: (B) *CDH1*, (C) *VIM*, (D) *CDH2*, (E) *TWIST*, (F) *ZEB1*, and (G) *SNAI2*. Gene expression levels were measured by qRT-PCR and normalized to *β-actin* ($2^{-\Delta\Delta Cq}$) (mean ± SD, $n = 3$). *$p < .05$ (ANOVA).

No. 3 was similar as to HBEC3 (Figure 2C). Significantly altered expression of *VIM* was measured in all clones compared with HBEC3. Whereas the expression of *VIM* was significantly higher in clone Nos. 1, 2, and 4, it was significantly lower in clone No. 3 compared with HBEC3 (Figure 2D). Only clone No. 1 showed significantly different expression of *TWIST1*, *ZEB1*, and *SNAI2* compared with HBEC3 (Figs. 2E–G). Clone No. 1 (as from here on termed T2-HBEC3) showed the clearest indications of EMT based on the expression of these EMT-marker genes, and was selected for further studies of DEP-induced transformation and toxicity. HBEC3 possess an epithelial morphology (Figure 3A), and T2-HBEC3 have a larger, more spindle-shaped mesenchymal/fibroblasts-like morphology (Figure 3B). Downregulation of E-cadherin and induction of vimentin proteins in T2-HBEC3 was also evident by immunoblotting (Figure 3C).

Migratory and Invasive Potential of HBEC3 and T2-HBEC3

A scratch wound closure assay was performed with HBEC3 and T2-HBEC3. Interestingly, T2-HBEC3 displayed a significantly slower migration pattern compared with HBEC3 (Figure 3D). HBEC3 completely closed the wound after 6 h, whereas T2-HBEC3 closed the wound after 48 h. Neither HBEC3 (data not shown) nor T2-HBEC3 (Figure 3E) had the capability to invade a reconstituted matrigel during the 48 h observation period.

Gene Expression Profiling

First, gene expression profiling was carried out on baseline samples of HBEC3 and T2-HBEC3 to explore intrinsic differences between the 2 cell lines. 429 genes (224[↑] and 205[↓]) were found to be significantly deregulated between HBEC3 and T2-HBEC3 and the 48 most significantly up- and down-regulated genes at baseline are presented in Table 1. Utilizing the ToppFun-tool, several of these genes were identified as being involved in i.e. regulation of cell migration and lung carcinogenesis: (*DNER*[↑], *FBLN1*[↑], *HBEGF*[↓], *IGFBP3*[↑], *LAMA4*[↑], *PROS1*[↑], *RAB25*[↓], *SPOCK1*[↑], *ST14*[↓], *TGFBR3*[↑], *TP53INP1*[↑], *CD9*[↓], *CLDN1*[↓], *DUSP6*[↓], *EPCAM*[↓], *EPHA1*[↓], *FOXA2*[↓], *HAS3*[↓], *HTRA1*[↑], *MUC1*[↑], *PMEPA1*[↑], *TIMP2*[↑], *EGR1*[↓], *EPHA1*[↓], *IL1B*[↓], and *VIM*[↑]).

At baseline there were 8 significantly deregulated pathways (KEGG database) between HBEC3 and T2-HBEC3 having particular implications for *in vitro* carcinogenesis: "*Axon guidance*", "*Focal adhesion*" and the "Mitogen-activated protein kinase (MAPK)-", "*Insulin-*", "*Toll-like receptor-*", "*TGF-β-*", "*Hedgehog-*", and "The mechanistic target of rapamycin (mTOR)-" signaling. A complete list of significantly deregulated pathways at baseline is presented in Supplementary Table 2A.

Next, to assess differences in toxic effects of DEP between the parental HBEC3 and T2-HBEC3, gene expression profiling was carried out on selected samples from DEP short-term exposure experiments. This included exposure to 50 or 200 μg DEP/ml for 48 h, and 200 μg DEP/ml for 24 or 72 h. In the dose-response experiments, more genes were deregulated after exposure to the highest concentration (200 μg DEP/ml) compared with 50 μg DEP/ml for both cell lines (Figure 4A). 16 genes were deregulated at both DEP-concentrations in HBEC3, while in T2-HBEC3 there were 15 common deregulated genes (Figure 4B). It is interesting to note that the only gene that was altered by the 2 DEP-exposures in both cell lines is *CYP1B1*. In the time-course experiment, more genes were deregulated after 72 h than after 24 h of exposure (200 μg DEP/ml) for both cell lines (Figure 4C). In total 3 genes were deregulated at both exposure times in HBEC3, while in T2-HBEC3 there were 4 common deregulated genes (Figure 4D).

A comprehensive overview of significantly affected pathways from DEP short-term exposure experiments is presented in Supplementary Tables 2B–I. Pathways significantly deregulated at 2 or more exposure scenarios (ie, in either experiment) are presented in Table 2. A full list of deregulated genes associated with each pathway at the different exposure scenarios is presented in Supplementary Table 3.

Four pathways were commonly deregulated in both HBEC3 and T2-HBEC3 in the short-term DEP-exposure experiments: "*Tryptophan metabolism*", "*Valine, leucine and isoleucine degradation*", "*Terpenoid backbone biosynthesis*" and "*Steroid biosynthesis*". Three pathways were significantly deregulated in HBEC3, only: "*Metabolism of xenobiotics by cytochrome p450*", "*Phagosome*", and "*Aldosterone-regulated sodium reabsorption*". In T2-HBEC3, several pathways associated with inflammatory responses were identified in addition to "*Synthesis and degradation of ketone bodies*", "*Butanoate metabolism*", and "*Pyruvate metabolism*".

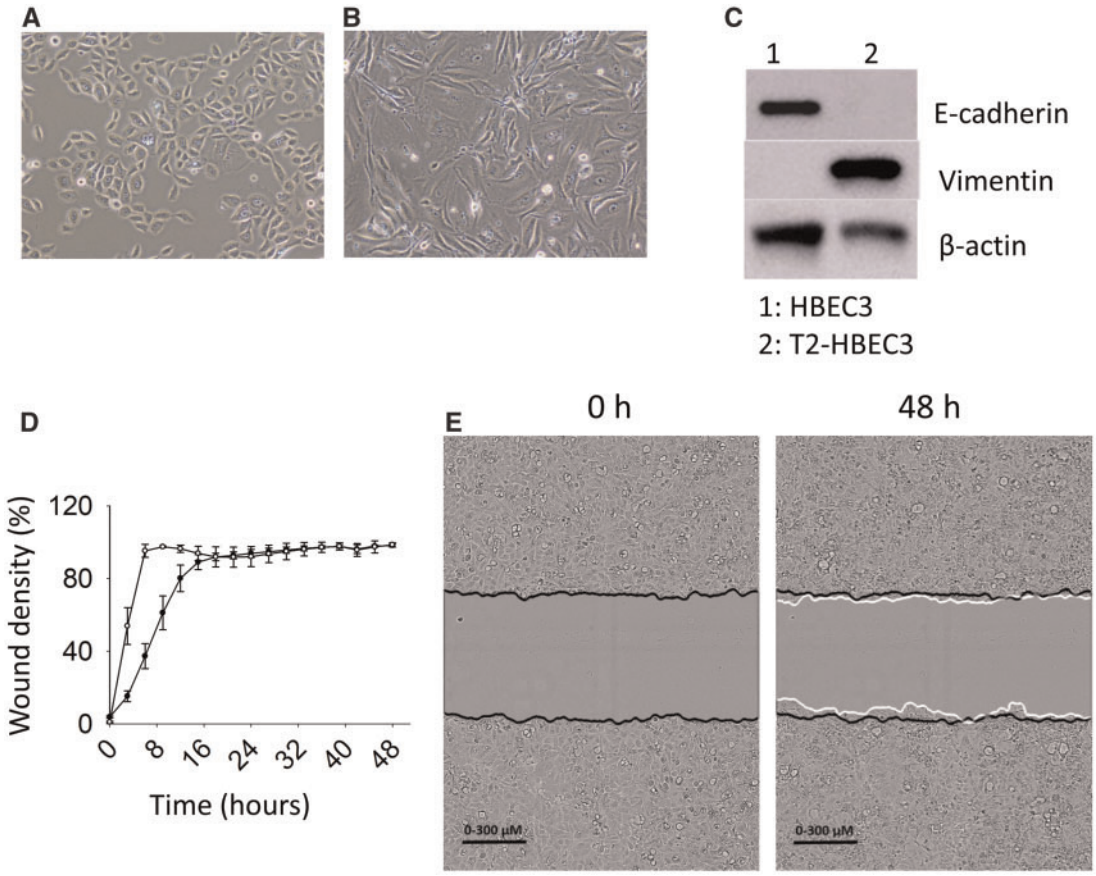


Figure 3. Morphology, and migratory and invasive potential. Light microscopy images (×10 magnification) of HBEC3 (A) and T2-HBEC3 (B). C, E-cadherin and vimentin protein levels were analyzed in HBEC3 and T2-HBEC3 by immunoblotting. β-actin was used as loading control. Migration and invasion were measured in a scratch wound closure assay. D, HBEC3 (○) and T2-HBEC3 (•) display significantly different migration curves. Data display wound density (percent) ± SE, $n = 3$. $p < 0.05$ (non-linear mixed effects model). E, Invasive potential of T2-HBEC3 analyzed by live cell imaging. Black lines display the original scratch wound made in the cell layer at 0 h and the white lines display the invading front of cells after 48 h.

Expression of CYP1A1, CYP1B1, and IL1B

Selected genes involved in xenobiotic metabolism and inflammation were measured by RT-qPCR in HBEC3 and T2-HBEC3 from the DEP short-term exposure experiments. In the dose-response experiment, gene expression of *CYP1A1* increased significantly for both cell lines starting at the lowest concentration of 25 μg DEP/ml (Figure 5A). For concentrations ranging between 50 and 400 μg DEP/ml, significantly higher induction of *CYP1A1* was observed for T2-HBEC3 compared with HBEC3. Expression of *CYP1A1* increased significantly with time for both cell lines (Figure 5B), but for HBEC3 induction was only statistically significant after 48 and 72 h. Induction of *CYP1B1* was also found in the short-term exposure experiments (Supplementary Figure 1A).

By gene expression profiling, *IL1B* was among the genes showing the most differential expression between HBEC3 and T2-HBEC3 at baseline (Table 1). When measured by RT-qPCR, HBEC3 showed generally higher expression of *IL1B* than T2-HBEC3 (Figure 5C). Expression of *IL1B* increased dose-dependently for both cell lines at the 3 highest exposure concentrations (100–400 μg DEP/ml). In the time-course experiment, significantly increased expression of the *IL1B* gene was found for both cell lines at all time-points (200 μg DEP/ml), but no differences between the cell lines were observed (Figure 5D). Higher levels of IL-1β protein were identified at all concentrations (0–400 μg DEP/ml) in culture media from HBEC3 compared with T2-HBEC3 (Figure 5E). Induction of *IL1A* was also found in the short-term DEP-exposure experiments (Supplementary Figure 1B). In addition, the *IL-6*, *IL-8* and *TNF-α* genes were measured, but their levels of expression were at or below the detection limit.

Analysis of DNA Strand Breaks

DNA strand breaks/alkali-labile sites were measured in HBEC3 and T2-HBEC3 exposed to 0, 25, 50, and 100 μg DEP/ml for 24 h. Significantly increased levels in DNA strand breaks were observed for both cell lines at all DEP concentrations, but there were no indications of dose-response relationships (Figure 5F). Increased oxidative DNA-damage associated with FPG sensitive sites was not observed (data not shown).

Effects of DEP-Exposure on Cellular Secretion of Eicosanoids

The effect of DEP-exposure on release of AA and several AA metabolites (Supplementary Table 4) into cell culture media was measured. Although the levels of lipoxygenase metabolites (5-, 8-, 12-, and 15-HETE) and LXA4 secreted into the growth medium were not changed (data not shown), AA and PG production was significantly affected by exposure to DEP. Secretion of AA was significantly higher in T2-HBEC3 than in HBEC3 at all concentrations (0–400 μg DEP/ml) in the dose-response experiments (Figure 6A). With HBEC3, but not T2-HBEC3, there was a reduction of secreted AA at the exposure concentrations 25–400 μg

Table 1. The 48 Most Significantly Deregulated Genes Between HBEC3 and T2-HBEC3 at Baseline

Symbol	log2FC	Symbol	log2FC
VIM	6.33	LAD1	–2.04
CPA4	4.26	CD9	–2.05
FBLN1	4.05	REPIN1	–2.06
SRGN	3.78	KRT18P13	–2.08
IGFBP3	3.77	DUSP6	–2.12
LOX	3.50	ST14	–2.13
CA9	3.45	EPHA1	–2.14
GAS1	3.44	CLDN1	–2.18
SVEP1	3.00	HBEGF	–2.21
SAMSN1	2.94	FOXA2	–2.22
CYBRD1	2.94	EGR1	–2.24
DKK1	2.89	TMEM30B	–2.25
SPOCK1	2.87	RAB25	–2.40
MXD4	2.85	UPP1	–2.41
MME	2.81	FGFBP1	–2.62
PROS1	2.64	HAS3	–3.14
HTRA1	2.49	IL1B	–3.82
SEZ6L2	2.48	EPCAM	–4.55
DHRS9	2.32		
DNER	2.31		
MUC1	2.31		
LAMA4	2.31		
PMEPA1	2.18		
TP53INP1	2.17		
TIMP2	2.14		
COL8A1	2.14		
TGFBR3	2.11		
NNMT	2.09		
MFGE8	2.09		
RPS6KA2	2.08		

The table display the 48 most significantly deregulated genes between baseline HBEC3 and T2-HBEC3 identified from gene expression profiling.
$p < 0.001$ and log2FC < -2.0 and > 2.0 (moderated *t*-statistics).

DEP/ml, compared with control. In the time-course experiment, exposure to 200 μg DEP/ml resulted in a reduction in AA secretion at all time-points from HBEC3 and after 48 and 72 h from T2-HBEC3, compared with the respective controls (Figure 6A).

PGs PGE_2 and $PGF_{2\alpha}$ were the major AA metabolites identified. Secretion of PGE_2 and $PGF_{2\alpha}$ (Figs. 6B and 6C) increased dose-dependently for HBEC3 only, in the range of exposure concentrations from 100 to 400 μg DEP/ml. Generally, higher levels of PGE_2 and $PGF_{2\alpha}$ secretion were found from T2-HBEC3 compared with HBEC3. PGE_2 secretion increased significantly after DEP-exposure in HBEC3 at all time-points (200 μg DEP/ml) compared with the respective controls. At 72 h of exposure, a higher increase in PGE_2 secretion was found in HBEC3 (4-fold) compared with T2-HBEC3 (<2-fold). Whenc compared with controls, there was increased secretion of $PGF_{2\alpha}$ from DEP-exposed (200 μg/ml) HBEC3 at all time-points. At 24 h, an increase (2-fold) of $PGF_{2\alpha}$ secretion was found in HBEC3 whereas with T2-HBEC3 a minor reduction was observed.

In accordance with the observed increase in PGE_2, PGA_2, which is a product of subsequent nonenzymatic 15-oxidation and 13, 14-reduction of PGE_2, was also measured. Unchanged levels of PGA_2 were found from T2-HBEC3 only, in the dose-response experiment. In the time-course experiment, significantly increased PGA_2 levels were identified in both cell lines. However, 1 or 2 orders lower concentrations of PGA_2 were found, compared with PGE_2 and $PGF_{2\alpha}$ (Supplementary Figure 3A). Secretion of 13, 14-DH-15-keto-PGE_2, the downstream product of $PGF_{2\alpha}$, decreased significantly for T2-HBEC3 at the highest exposure (400 μg DEP/ml), meanwhile it did not change for HBEC3 at any concentration (Figure 6D). 13, 14-DH-15-keto-PGE_2 secretion levels were reduced with time after 200 μg DEP/ml for HBEC3 after 48 and 72 h compared with the respective controls.

Levels of PGI_2 and PGD_2, ie, products of 2 other AA metabolic pathways, were not modulated by DEP (data not shown); however, a significant increase in release of PGJ_2 (a direct downstream metabolite of PGD_2) was found in HBEC3 at 50–400 μg DEP/ml, compared with control (Supplementary Figure 3B), suggesting that also the alternative PG synthase pathway is induced in HBEC3. Interestingly, no PGJ_2 was identified in T2-HBEC3.

Importantly, the levels of 8-iso-$PGF_{2\alpha}$, a biomarker of membrane lipid peroxidation, increased significantly for HBEC3 at the 3 highest exposure concentrations (100–400 μg DEP/ml) (Figure 6E). Higher levels of 8-iso-$PGF_{2\alpha}$ were found in T2-HBEC3 compared with HBEC3 at 0, 25, and 400 μg DEP/ml. Secreted levels of 8-iso-$PGF_{2\alpha}$ increased with time for T2-HBEC3 only.

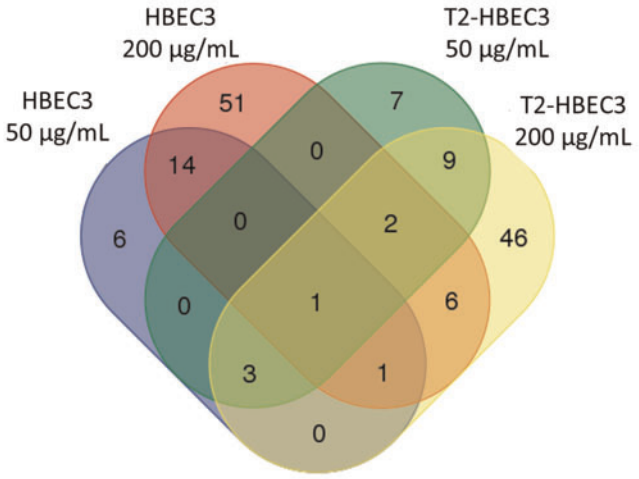
Identification of Deregulated Genes Involved in AA Metabolism

Based on the results from eicosanoid secretion, gene expression profiling data was explored to look for genes that could aid in explaining the observed changes in AA metabolism. In T2-HBEC3 compared with HBEC3 exposed to 200 μg DEP/ml for 24 h, deregulation of *PLA2G2A*(↑), *PLA2G10*(↑), *ALOX5*(↑), *CYP2J2*(↑), *LTA4H*(↑), *AKR1C3*(↑) was found (Supplementary Table 2H). This pathway was also deregulated in T2-HBEC3 compared with HBEC3 after 48 h at all exposure concentrations (50–200 μg DEP/mL) (Supplementary Table 2F and G). In T2-HBEC3 compared with HBEC3 at 50 μg DEP/ml *ALOX5*(↑), *CYP2J2*(↑), *PTGES*(↑), *PLA2G2A*(↑), *COX-2*(↑), *CBR3*(↓), *GPX3*(↓), and *CYP4F11*(↓) were deregulated, meanwhile at 200 μg DEP/ml *ALOX5*(↑), *CYP2J2*(↑), *PLA2G2A*(↑), *COX-2*(↑), *GPX3*(↓), and *GPX2*(↓) were found to be altered. Verification of selected DEGs by RT-qPCR is found in Supplementary Figure 2.

DISCUSSION

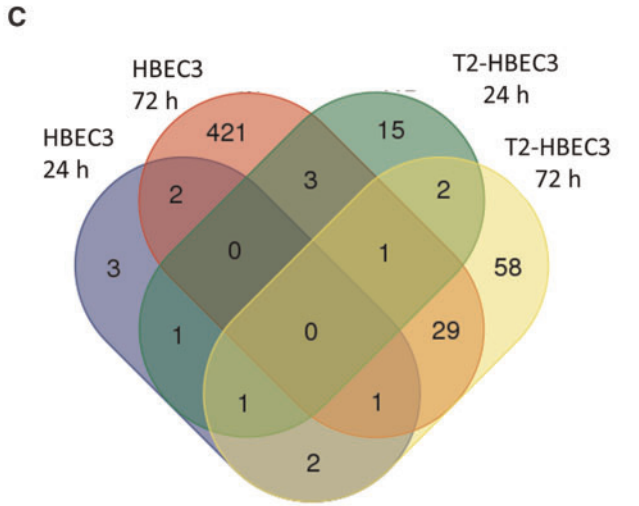
This study reports that DEP (NIST SRM2975) transformed HBEC3 *in vitro*. Transformed clones showed varying degree of expression of EMT-markers. One clone, T2-HBEC3, gave marked indications of EMT and showed differences in baseline gene expression profiles when compared with parental HBEC3. T2-HBEC3 also showed altered sensitivity to short-term DEP-exposure regarding gene expression profiles and inflammatory markers.

Long-term DEP-exposed HBEC3 formed a significantly increased number of anchorage-independent colonies compared with unexposed cells. The present DEP *in vitro* transformation study is of particular importance as HBEC3 are genomically stable with intact tumor suppressor TP53 checkpoint, and show a low rate of spontaneous transformation (Damiani *et al.*, 2008; Ramirez *et al.*, 2004). The TE of 0.39% was low. A previous study with HBEC1 and HBEC2 exposed to methylnitrosourea and B[*a*]P-diol-epoxide-1 reported a TE of 0.2%–3.0% (Damiani *et al.*, 2008). B[*a*]P and several chemical species in CSC are present in various concentrations in DEP. However, this is the first time, to our knowledge, an immortalized HBEC line with a normal phenotype has been stably transformed following *in vitro* exposure to intact DEP with low content of organic compounds. The carcinogenic potential of DEP is debated (HEI Diesel Epidemiology Panel, 2015), highlighting the need for further studies



B

	HBEC3			T2-HBEC3	
	50 µg/mL	200 µg/mL		50 µg/mL	200 µg/mL
Genes	log2FC	log2FC	Genes	log2FC	log2FC
CYP1A1	2.67	2.23	INSIG1	1.71	2.47
CYP1B1	2.55	2.64	CYP1B1	2.30	2.78
RNY4	1.66	1.28	SC4MOL	1.85	2.33
EREG	1.47	0.75	SCD	1.44	1.72
ACSS2	1.24	1.49	LDLR	1.28	1.78
LIPG	1.10	0.73	SQLE	1.20	1.70
ITGB5	0.94	1.63	IER3	1.10	2.17
F3	0.90	1.76	FTH1	1.00	0.93
STC1	0.87	0.85	FDFT1	0.85	1.50
HMGCR	0.71	1.02	MTE	0.85	1.47
ABCC3	0.66	1.40	DKK3	0.64	1.06
ADRB2	-0.76	-1.32	EFEMP1	-0.75	-0.94
GLUL	-0.78	-1.47	ALDH1A1	-0.76	-1.05
DNAJC15	-1.00	-1.32	SEPP1	-0.96	-1.53
EPSTI1	-1.38	-1.60	C10orf99	-1.08	-1.99
CD24	-1.44	-2.10			



D

	HBEC3			T2-HBEC3	
	24 h	72 h		24 h	72 h
Genes	log2FC	log2FC	Genes	log2FC	log2FC
RNY1	0.58	1.40	SLC29A2	0.59	0.70
LDLR	0.77	1.83	SQLE	1.10	1.67
STOM	-0.68	-0.94	PNP	0.82	0.65
			ABCC3	0.69	1.14

Figure 4. Significantly deregulated genes in the short-term DEP-exposure experiments. A, Venn diagram display statistically significant deregulated genes between HBEC3 and T2-HBEC3 exposed to 50 and 200 µg/ml DEP for 48 h and (C) Venn diagram display statistically significant deregulated genes between HBEC3 and T2-HBEC3 exposed to 200 µg/ml DEP for 24 and 72 h. $p < .001$ and log2FC < -0.58 and > 0.58 (Moderated *t*-statistics). (B) Heat maps display common deregulated genes within each cell line between the 2 exposure concentrations while (D) heat maps display common deregulated genes within each cell line between the 2 time-points. Red and green indicates up- and downregulation of gene expression, respectively.

investigating the effects of not only DEP-extracts, but also of intact particles with all its constituents.

All DEP-transformed clones showed significantly reduced *CDH1* expression, accentuating the importance of E-cadherin downregulation in EMT. With the exception of clone No. 3, the clones showed increased expression of *CDH2* and *VIM*. Thus, variability in expression of EMT-marker genes between the transformed clones was found. EMT is a plastic process and cancer cells of epithelial origin may pass through EMT to various extent, where some cells may retain particular epithelial traits, whereas others may become fully mesenchymal (Kalluri and Weinberg, 2009). The present data underline that clones with different patterns of expression of EMT-marker genes can have the capacity to grow anchorage-independently in soft agar.

Significantly increased expression of *TWIST1*, *ZEB1*, and *SNAI2* was only measured in T2-HBEC3. T2-HBEC3 also displayed the highest increase in vimentin, compared with the other clones. Vimentin is important in cytoskeleton organization and cellular mechanical strength in cancers of epithelial origin. Thus, induction of vimentin may have contributed to the change towards a mesenchymal/fibroblast-like morphology (Liu *et al.*, 2015). T2-HBEC3 showed reduced migration compared with HBEC3. Generally, increased cellular capacity to migrate and invade coincides with EMT. However, altered migration and

invasive potential may be uncoupled events in EMT (Schaeffer *et al.*, 2014). It was shown that exposure to DEP-induced disruption of cell-polarity and focal adhesion remodeling in alveolar epithelial cells leading to disruption of directional migration (LaGier *et al.*, 2013). A recent study also reported reduced migratory potential for CSC-transformed HBEC2 (Bersaas *et al.*, 2016). However, in contrast to CSC-transformed HBEC2, the DEP-transformed T2-HBEC3 did not invade a reconstituted basement membrane, suggesting that these cells represent a model of early steps in carcinogenesis.

Table 2. Significantly Deregulated Pathways in the Short-Term DEP-Exposure Experiments

Deregulated Pathways
Common deregulated pathways[a]
Tryptophan metabolism
Valine, leucine and isoleucine degradation
Terpenoid backbone biosynthesis
Steroid biosynthesis
Deregulated pathways in HBEC3[b]
Metabolism of xenobiotics by cytochrome P450
Phagosome
Aldosterone-regulated sodium reabsorption
Deregulated pathways in T2-HBEC3[c]
Synthesis and degradation of ketone bodies
Malaria
Butanoate metabolism
Rheumatoid arthritis
Pyruvate metabolism
Graft-versus-host disease
Rheumatoid arthritis

Data are from the dose-response and time-course experiments combined. Pathways have adjusted p-value (Holm) $< .01$.
[a]Pathways (Goemans's global test and the KEGG database) deregulated in both HBEC3 and T2-HBEC3.
[b]Pathways deregulated in HBEC3 only.
[c]Pathways deregulated in T2-HBEC3 only.

Results from baseline gene expression profiling revealed deregulation of several pathways in T2-HBEC3 related to carcinogenesis. Originally identified as guidance for axons during central nervous system development, molecules belonging to semaphorins and ephrins are now appreciated as contributors in lung carcinogenesis (Nasarre *et al.*, 2010). Alterations in integrins and structural constituents of extracellular matrix may participate in altering proliferative, migratory and apoptotic signals mediated by ie, phosphatidylinositol 3-kinase and MAPK (Paoli *et al.*, 2013). These pathways together with mTOR are frequently deregulated in lung carcinogenesis through alterations in genes coding for key components of the cascades or cell-surface receptors (De *et al.*, 2012; Ekman *et al.*, 2012).

Gene expression profiling from short-term DEP-exposures revealed deregulation of *"Tryptophan metabolism"*, *"Valine, leucine and isoleucine degradation"*, *"Terpenoid backbone biosynthesis"*, and *"Steroid biosynthesis"* pathways in both HBEC3 and T2-HBEC3. In addition, DEP specifically induced deregulation of pathways related to particle uptake and xenobiotic metabolism in HBEC3, whereas pathways related to inflammation and metabolism were affected in T2-HBEC3. Microarray analysis of A549 exposed to Milan urban air winter $PM_{2.5}$ revealed deregulated pathways involved in ie, xenobiotic metabolism, inflammation and lipid metabolism, while summer $PM_{2.5}$ preferentially affected pathways involved in cell signaling, -function, and -assembly (Gualtieri *et al.*, 2012). In BEAS-2B, Milan urban PM showed regulation of

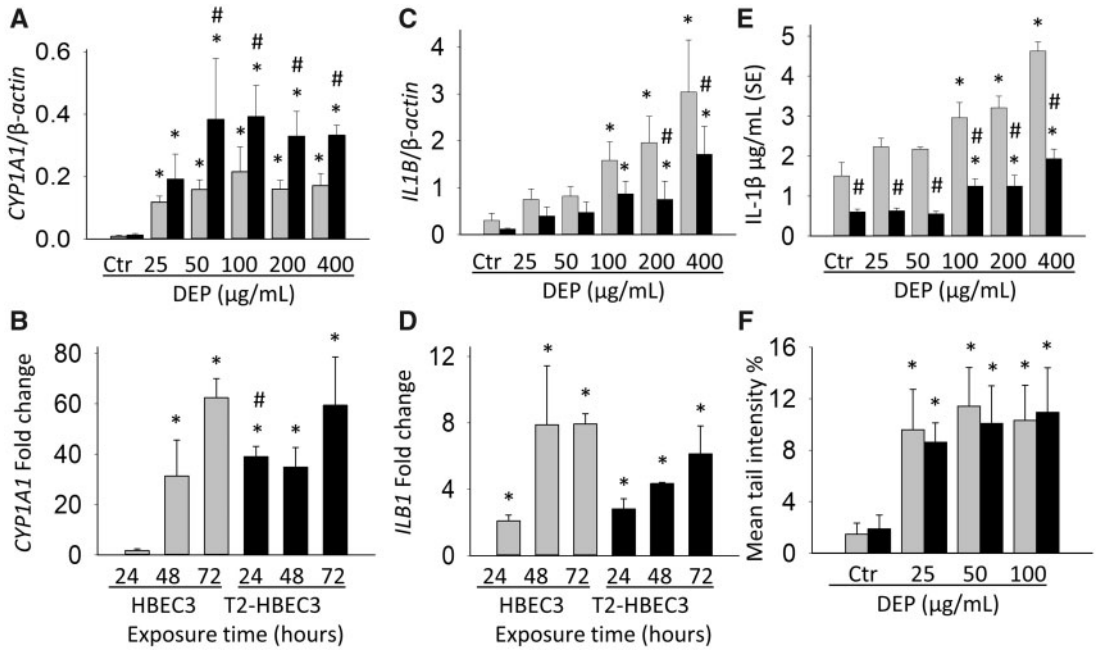


Figure 5. Gene expression of *CYP1A1* and *IL1B*, and analysis of DNA strand breaks by Comet assay. HBEC3 in gray bars and T2-HBEC3 in black bars. Gene expression of *CYP1A1* (A) and *IL1B* (C) in HBEC3 and T2-HBEC3 from the 48 h dose-response experiment and *CYP1A1* (B) and *IL1B* (D) from the time-course experiment with 200 μg DEP/mL. Gene expression levels for the dose-response experiment (Figs. 5A and 5C) were measured by RT-qPCR and normalized to *β-actin* ($2^{-\Delta\Delta Cq}$) (mean ± SD, $n = 3$). For the time-course experiment (Figs. 5B and 5D), gene expression levels were normalized to *β-actin* and the corresponding unexposed control at the given time-point. E, Protein levels of IL-1β release to cell culture media from HBEC3 and T2-HBEC3 were measured by ELISA in the dose-response experiment. * and #: $p < .05$ (linear mixed effects model). *Statistically significant differences between control to concentration/time DEP. #Statistically significant differences between HBEC3 and T2-HBEC3 at corresponding exposure concentrations/time. F, DNA strand breaks were measured by alkali Comet assay in a dose-response experiment with HBEC3 and T2-HBEC3. Data display mean % tail DNA ± SD, $n = 3$. *$p < .05$ (Log-transformed data, ANOVA Dunnett's post hoc test).

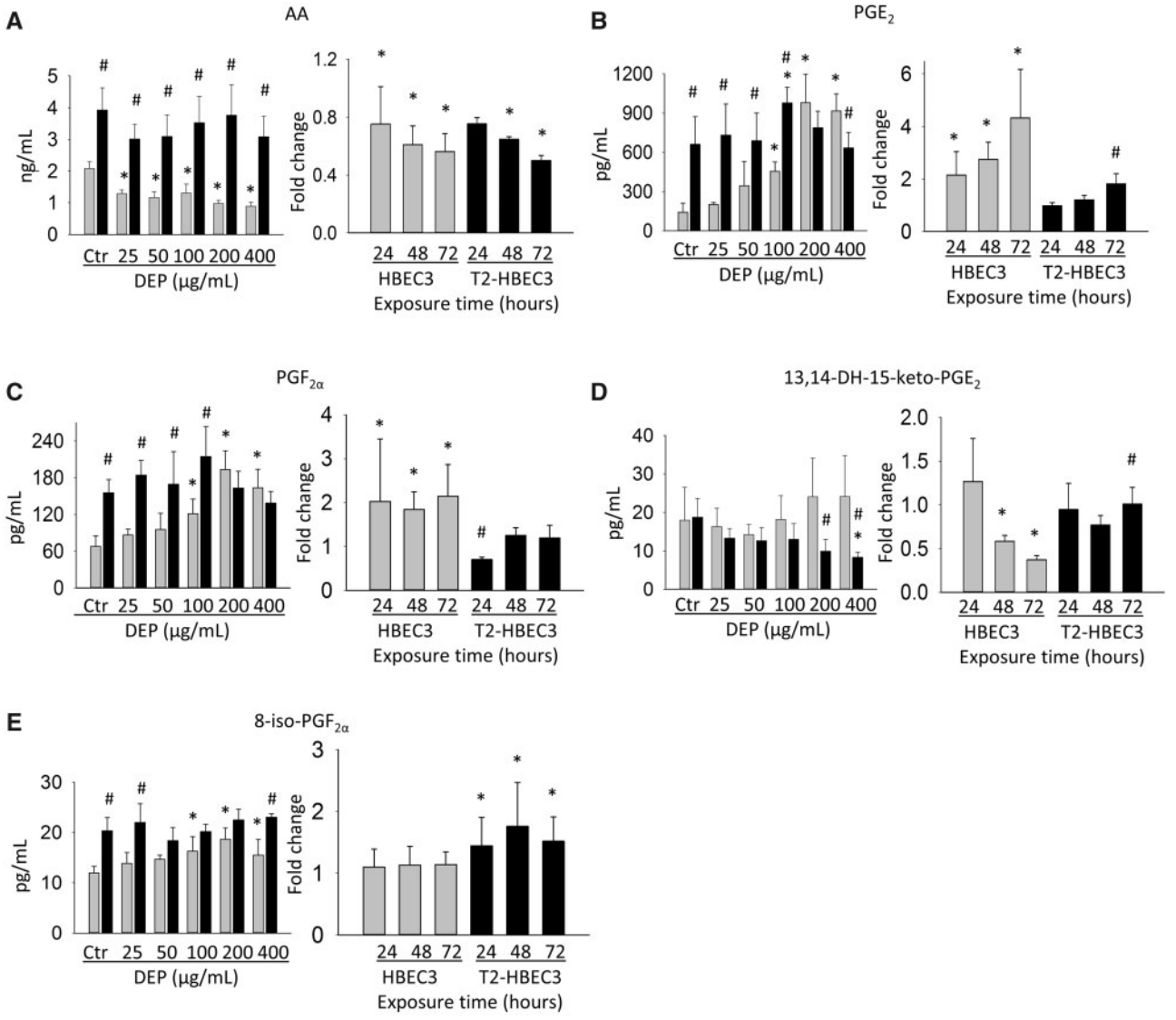


Figure 6. Release of AA and PGs to cell culture media in the short-term DEP-exposure experiments. HBEC3 in gray bars and T2-HBEC3 in black bars. (A) AA, (B) PGE_2, (C) $PGF_{2\alpha}$, (D) 13, 14-DH-15-keto-PGE_2, (E) 8-iso-$PGF_{2\alpha}$. Data display mean ± SD, $n = 3$. Eicosanoid concentration measured from the dose-response experiment is presented in pg/ml or ng/ml, while those from the time-course experiment are presented as fold change compared with the corresponding unexposed control at the given time-point. * and #: $p < .05$ (linear mixed effects model). *Statistically significant differences between control to concentration DEP and between control to exposure time. #Statistically significant differences between HBEC3 and T2-HBEC3 at corresponding exposure concentrations and at corresponding time-points.

pathways involved in oxidative stress, inflammation and DNA-damage responses (Longhin *et al.*, 2016). Thus, exposure to DEP and urban PM with different particle and chemical characteristics appears to elicit deregulation of several similar pathways.

Microarray analysis of BEAS-2B exposed to DEP and biodiesel particle extracts also showed deregulation of *"Metabolism of xenobiotics by cytochrome p450"*, *"Metabolism of lipids and lipoproteins"*, and *"Steroid biosynthesis"*, in addition to other pathways, ie, related to bile acid synthesis (Libalova *et al.*, 2016). Most deregulated pathways may be linked to aryl hydrocarbon receptor (AHR) signaling and probably be explained by differences in particle PAH levels (Gualtieri *et al.*, 2012). Accordingly, PAH was reported to be central for the toxic effects of SRM1649a extracts, via its AHR dependent mutagenic and non-genotoxic effects (Andrysik *et al.*, 2011).

Associations between the tryptophan metabolite kynurenine, inflammation and cancer have been suggested (Opitz *et al.*, 2011). Degradation of valine, leucine, and isoleucine generates propionyl-CoA and/or acetyl-CoA utilizable for lipid synthesis. Lung lipid homeostasis is a tightly regulated process and its disruption can cause inflammation participating in lung injury (Plantier *et al.*, 2012). The *"Mevalonate (MVA) pathway"* generates isoprenoids, being key metabolites for *de novo* cholesterol and steroid synthesis (Goldstein and Brown, 1990). A link between inflammation, carcinogenesis and the MVA pathway has been reported (Karlic *et al.*, 2015; Steffens and Mach, 2004). Dysregulation of small GTPases of the Ras family is important in carcinogenesis, and their activation depends on the addition of isoprenyl (Konstantinopoulos *et al.*, 2007). Regulation of *"Terpenoid backbone biosynthesis"* may represent a mechanism for altered posttranscriptional modification of such proteins. *"Steroid biosynthesis"* is linked to the MVA pathway and emerging evidence from both epidemiological and experimental studies indicate correlations with

steroid hormones and human nonsmall-cell lung cancer progression (Kazmi *et al.*, 2012). Together, deregulation of these pathways may participate as "routes of action" for detrimental effects of DEP.

Short-term exposure to DEP induced expression *CYP1A1* and *CYP1B1* (RT-qPCR) in both HBEC3 and T2-HBEC3 despite low content of organic carbon compounds in SRM2975. Metabolic activation of PAH by the cytochrome P450 family can lead to early initiating events in carcinogenesis (Shimada and Fujii-Kuriyama, 2004), and *CYP1A1* may constitute a sensitive biomarker for DEP-induced effects (Totlandsdal *et al.*, 2010). Higher induction of *CYP1A1* was found in T2-HBEC3 compared with HBEC3. *CYP1A1* was also induced at an earlier time-point in T2-HBEC3, indicating a greater xenobiotic response to DEP in the transformed cells. *CYP1B1* followed a similar pattern. Significantly higher levels of *IL1A* and *IL1B* gene expression, and IL-1β secretion were measured from HBEC3 compared with T2-HBEC3 after DEP-exposure. IL-1β is critical for DEP-induced pulmonary inflammation (Provoost *et al.*, 2011). In accordance with earlier findings (Arlt *et al.*, 2015; Totlandsdal *et al.*, 2010), our data support an inverse relationship between induction of CYP-enzymes and inflammation. Combined, this may indicate a downregulation of inflammatory responses in T2-HBEC3 that could represent a way to increase cell survival after DEP-exposure.

Increased single strand breaks/alkaline-labile sites were observed after DEP-exposure, while oxidative DNA-damage was unchanged. Similar results were found in BEAS-2B exposed to Milan winter $PM_{2.5}$ (Gualtieri *et al.*, 2011). In contrast, exposure of A549 to SRM2975 increased oxidative DNA-damage (Jantzen *et al.*, 2012). This discrepancy may be related to experimental setup, including intrinsic differences between cell lines. Accordingly, lack of induction of oxidative DNA-damage by SRM1650b extract was also reported in liver, pulmonary, and prostate cell lines (Pálková *et al.*, 2015), suggesting that increased formation of reactive oxygen species (ROS) may not always contribute to DEP-induced DNA-damage.

Higher secretion of AA from T2-HBEC3 may be linked to induction of genes belonging to the phospholipase A2 family (Supplementary Table 2). PGE_2 and $PGF_{2\alpha}$, the major secreted AA metabolites, increased dose-dependently for HBEC3, whereas generally higher and unchanged levels were measured for T2-HBEC3. Similar results were obtained from the time-course experiment where higher levels of PGE_2 and $PGF_{2\alpha}$ release after DEP-exposure were observed for HBEC3. The generally higher *PTGES* (PG E synthase, Supplementary Figure 2E) and PGE_2 levels, in addition to reduced E-cadherin found in T2-HBEC3, are in compliance with previous results showing inverse association between decreased E-cadherin levels and increased PGE_2 synthesis (Brouxhon *et al.*, 2007). DEP-induced PGE_2 and $PGF_{2\alpha}$ secretion has been reported from canine AM and in bronchoalveolar lavage fluid (BALF) from mouse and rat (Alessandrini *et al.*, 2009; Beck-Speier *et al.*, 2005; Henderson *et al.*, 1988). Higher baseline levels of *COX-2* were measured in T2-HBEC3, which may aid in explaining increased secretion of several eicosanoids. Interestingly, no dose-response relationship of *COX-2* induction was found following DEP-exposure for either cell line. However, a significant increase with time was found for HBEC3 (Supplementary Figure 2D).

Levels of 8-iso-$PGF_{2\alpha}$ increased dose-dependently in HBEC3 indicating increased lipid peroxidation. Secretion of 8-iso-$PGF_{2\alpha}$ increased with time in T2-HBEC3. Generally higher levels of 8-iso-$PGF_{2\alpha}$ measured in T2-HBEC3 after DEP-exposure indicates continuous lipid peroxidation; a trait commonly found in cancer cells showing increased ROS production (Toyokuni *et al.*, 1995). Generation of 8-iso-$PGF_{2\alpha}$ after DEP-exposure has been found in canine AM and BALF from mouse (Alessandrini *et al.*, 2009; Beck-Speier *et al.*, 2005). Combined, these data indicate that HBEC3 elicits a greater inflammatory response to short-term DEP-exposure meanwhile T2-HBEC3 is constitutively sensitized, thus, potentially having implications for DEP-induced inflammation and carcinogenesis.

Results indicated cellular uptake of DEP mainly concentrated around the cell nucleus, but particle agglomerates attached to the cell surface were also found. Uptake in epithelial cells may be mediated by actin-dependent phagocytosis (Boland *et al.*, 1999), which by itself can trigger biological responses. However, particles and/or adsorbed organic chemicals may also interact directly with cellular plasma membranes and elicit biological responses through ion channels and membrane and intracellular receptors (Øvrevik *et al.*, 2015).

SRM2975 represents a well characterized and widely used particle model of DEP (Klein *et al.*, 2017). Interestingly, these particles with a low concentration of organic compounds (approximately 2%), nevertheless induced transformation. As exposures to DEP in occupational settings can be considerable, the concentration used in the transformation study (100 μg/ml) may be considered biologically relevant (Benbrahim-Tallaa *et al.*, 2012; Li *et al.*, 2003). Also, this concentration has frequently been used in other mechanistic studies (Beck-Speier *et al.*, 2005; Jantzen *et al.*, 2012).

In conclusion, long-term DEP-exposure transformed HBEC3 *in vitro*. T2-HBEC3 acquired several early traits of carcinogenesis. Furthermore, HBEC3 and T2-HBEC3 show different baseline gene expression profiles and susceptibility to short-term DEP-exposure regarding genes involved in xenobiotic and lipid metabolism, as well as inflammation. This study adds information of immunomodulatory effect markers measured from DEP-exposure and differences between normal and sensitized bronchial epithelial cells of the human lung.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

ACKNOWLEDGMENTS

The authors wish to thank Ms Rita Bæra for skillful technical assistance.

FUNDING

This study was supported by the Czech Science Foundation (grant No. 14-22016S to J.N., E.H., and M.M.; P503-12-G147 to K.V. and H.L.) and by the Ministry of Youth, Education and Sports of the Czech Republic (LO1508 to P.R. and. LM2015073 to J.T.).

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