RESEARCH ARTICLE

Controlled human exposures to diesel exhaust: a human epigenome-wide experiment of target bronchial epithelial cells

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Abstract

Diesel exhaust (DE) is a major contributor to ambient air pollution around the world. It is a known human carcinogen that targets the respiratory system and increases risk for many diseases, but there is limited research on the effects of DE exposure on the epigenome of human bronchial epithelial cells. Understanding the epigenetic impact of this environmental pollutant can elucidate biological mechanisms involved in the pathogenesis of harmful DE-related health effects. To estimate the causal effect of short-term DE exposure on the bronchial epithelial epigenome, we conducted a controlled single-blinded randomized crossover human experiment of exposure to DE and used bronchoscopy and Illumina 450K arrays for data collection and analysis, respectively. Of the 13 participants, 11 (85%) were male and 2 (15%) were female, and 12 (92%) were White and one (8%) was Hispanic; the mean age was 26 years (SD = 3.8 years). Eighty CpGs were differentially methylated, achieving the minimum possible exact P-value of $P = 2.44 \times 10^{-4}$ (*i.e.* $2/2^{13}$). In regional analyses, we found two differentially methylated regions (DMRs) annotated to the chromosome 5 open reading frame 63 genes (*C5orf63*; 7-CpGs) and unc-45 myosin chaperone A gene (*UNC45A*; 5-CpGs). Both DMRs showed increased DNA methylation after DE exposure. The average causal effects for the DMRs ranged from 1.5% to 6.0% increases in DNA methylation at individual CpGs. In conclusion,

we found that short-term DE alters DNA methylation of genes in target bronchial epithelial cells, demonstrating epigenetic level effects of exposure that could be implicated in pulmonary pathologies.

Key words: diesel exhaust; respiratory health; epigenetics; DNA methylation; EWAS

Introduction

Outdoor air pollution is a leading cause of morbidity and mortality globally, contributing to 4.2 million premature deaths worldwide in 2016 [1]. A major contributor to air pollution is diesel exhaust (DE) produced from the combustion of diesel fuel, which represents a complex mixture of chemicals. More than 40% of on-road vehicles around the world use diesel for fuel, and the global consumption of diesel has increased by eightfold since 1970 [2]. Exposure to DE is ubiquitous, as it is produced from engines in on-road and off-road vehicles, heavy equipment, and power generators. In the European Union, on-road diesel vehicles contributed to approximately 60% of transportation-attributable emissions of particulate matter <2.5 microns in diameter ($PM_{2.5}$) in 2015 [3]. On a global scale, the population-weighted transportation-attributable fraction for annual average emissions of $PM_{2.5}$ and ozone (O_3) were 12% and 11%, respectively [3]. The widespread and growing use of diesel as fuel, especially in urban settings, poses a serious threat to public health due to its contribution to air pollution.

DE is a mixture of solid particulate matter, condensed components, and gases that are known to have negative health effects. The solid fraction of DE is primarily composed of elemental carbon (EC) and metals, and the gaseous components include non-toxic inorganic gases and organic molecules like polycyclic aromatic hydrocarbons [4]. Humans exposed to these compounds through inhalation of DE can experience adverse health effects both in localized areas of immediate exposure (i.e. the respiratory tract) and in peripheral organ systems. Exposure to DE can induce oxidative stress, DNA damage, direct cytotoxic effects, and pro-inflammatory signaling that impair the lungs [5–7]. Other organs can become affected due to the transepithelial transport of particles and gases and spread of inflammatory cytokines and biomolecules generated in the lung tissue through blood [4, 8, 9]. On a population-level, several epidemiology studies have found significant associations between occupational exposure to DE and the development of lung and bladder cancers [10–12]. Consequently, the International Agency for Research on Cancer (IARC) classified DE as a known human carcinogen (Group 1) based on sufficient evidence that exposure is associated with an increased risk for lung cancer [13].

The epigenome is at the intersection between genetics, environment, and cellular responsiveness. Epigenetics is defined as DNA modifications, other than to the DNA sequence itself, which are maintained in cell division with stable consequences to gene expression [14]. The epigenome has been shown to be sensitive to environmental toxicant exposure with the potential for both persistence and malleability of changes [15, 16]. The most widely studied epigenetic modification is the addition of a methyl (CH_3) group to cytosines within CpG dinucleotides. Epigenome-Wide Association Studies (EWAS) have been widely conducted in the last decade to study diseases and the effects of environmental exposures [17]. However, the majority of human EWAS performed to date face two major challenges. First, observational and cross-sectional epidemiological studies limit the interpretation of findings due to the lack of randomization.

Second, most studies analyze DNA from easily accessible tissues and fluids, such blood samples and buccal cell DNA. To overcome these two limitations, we conducted a controlled single-blinded randomized crossover experiment with human participants exposed to DE and clean air and collected target bronchial epithelial cells at each visit. Prior studies have shown that exposure to different air pollutants affects DNA methylation in both animal models and humans [18]. Therefore, we hypothesized that short-term DE would lead to changes in DNA methylation that are present within 24-h following exposure.

Materials and Methods

Study Design

The overall study design, sample, and methods have been described previously [19, 20]. Briefly, healthy participants were recruited under a contract with Westat Corporation (Rockville, MD, USA), Clinical Trial NCT01492517. All study participants were free of cardiopulmonary diseases and allergies, as determined by a detailed medical history, questionnaire, and physical examination. Participants were excluded if they were smokers or pregnant or had a forced vital capacity (FVC) or forced expiratory volume in the first second of expiration (FEV_1) $<80\%$ predicted for their height and age. Participants were informed of the procedures and potential risks and provided written signed informed consent. Controlled exposures were conducted at the US Environmental Protection Agency (EPA) Human Studies Facility on the campus of the University of North Carolina (UNC), Chapel Hill. The protocol and consent forms were approved by the UNC School of Medicine Committee on the Protection of the Rights of Human Subjects and the US EPA's Institutional Review Board.

A total of 13 healthy volunteers were randomized to three experimental conditions: DE, O_3 (0.300 ppm), and clean air (CA). Results from the O_3 exposure experiments have been previously published [20, 21]. The 13 healthy volunteers had a mean age (SD) of 26 years (3.8 years); 11 were male (84.6%), 2 were female (15.4%), 12 were White (92.3%), and one (3.9%) was Hispanic. Relevant to this analysis, six volunteers were exposed to DE at the first study visit, four at the second visit, and the remaining three during a third and final visit. Controlled CA exposure occurred at the first, second, and third study visits for 4, 3, and 6 of the participants, respectively (Table 1). Each study participant was exposed on separate occasions to clean air and up to $300\,\mu g/m^3$ of DE for 2 h with intermittent periods of exercise in a controlled environment chamber. A minimum washout period of 13 days between exposures was implemented. The exposures were conducted at the same time of the day and on the same day of the week. The exposure chambers were maintained at 40% relative humidity for all exposures. During the 2-h exposure period, participants alternated between 15 min of rest and 15 min of exercise on a cycle ergometer. The cycle ergometer workload was adjusted so that subjects breathed at a ventilatory rate, normalized for body surface area, of $25\,l\,min^{-1}\,m^{-2}$. DE was obtained from a diesel power generator and introduced

Table 1: Characteristics among participants ($n = 13$)

	n (%) or mean (SD)
Sex	
Male	11 (84.6%)
Female	2 (15.4%)
Race	
White	12 (92.3%)
Hispanic	1 (7.7%)
Age (year)	25.7 (3.8)
Visit order for diesel exposure	
First	6 (46.2%)
Second	4 (30.8%)
Third	3 (23.1%)
Visit order for clean air exposure	
First	4 (30.8%)
Second	3 (23.1%)
Third	6 (46.1%)

into the exposure chamber after dilutions with clean high-efficiency particulate air, charcoal-filtered air, and humidified air to obtain a chamber concentration of up to 300 µg/m^3.

DNA Methylation Measurements

Within 24-h post-exposure to either clean air or DE, study participants underwent a research bronchoscopy with brush biopsy to obtain bronchial epithelial cells as previously described and following a standard protocol [22, 23]. The cytology brushes containing epithelial cells tips were placed in a 1.5 ml tube with 200 µl Trizol Buffer (ThermoFisher Scientific). DNA was extracted using the Gentra Puregene Buccal Cell Kit (Qiagen, Inc.), and samples were stored frozen at −80°C before analysis. DNA extracted from the bronchial epithelial cells was sent to a commercial laboratory (Expression Analysis, Durham, NC, USA) for DNA methylation assessment using the Illumina HumanMethylation 450K BeadChip array. The extracted DNA samples were placed on four chips. We performed background correction using noob and dye bias correction as well as corrected for probe design bias arising from Type I and Type II probes with the Beta-mixture quantile normalization (BMIQ) method [24, 25]. The DNA methylation β-value, or the proportion of cytosine methylated, at 484 531 CpGs was used for analyses.

Statistical Analyses

The statistical analysis approach has been described previously [21]. Briefly, to estimate the average causal effect (ACE) of DE on the bronchial epithelial epigenome, we assumed no carry-over and no time effects and defined an estimate for the ACE: the mean of the observed participant methylation difference between diesel exposure and clean air for each CpG site (484 531 CpGs). For individual i at study visit j randomized to DE ($w_{i,j} = 1$) and clean air ($w_{i,j} = 0$), the observed DNA methylation difference at CpG k ($d_{i,k}$) is defined by the two measured outcomes:

- $d_{i,k} = Y_{i,j=2,k}(w_{i,j=2} = 1) - Y_{i,j=1,k}(w_{i,j=1} = 0)$ if the participant was exposed to clean air first, and
- $d_{i,k} = Y_{i,j=1,k}(w_{i,j=1} = 1) - Y_{i,j=2,k}(w_{i,j=2} = 0)$ if the participant was exposed to DE first.

We assume a Bernouilli assignment mechanism and calculate the univariate Fisher-exact P values based on the standard paired t-test statistic and the $2^{13}/2$ possible randomizations:

$$T_{k,paired} = |\ (1/13)\ \Sigma_{i=1:13} d_{i,k}|/\ [S_D/13^{1/2}],$$
$$\text{Where } S_D{}^2 = [1/13]\ [\Sigma_{i=1:13}\left(d_{i,k} - (1/13)\ \Sigma_{i=1:13} d_{i,k}\right)^2].$$

With this assignment mechanism and test statistic, the minimum possible observable P-value is $2/2^{13}$ or $P = 2.44 \times 10^{-4}$. It has been recommended to analyze randomized trials using the exact null randomization distribution, which, in small samples, can substantially differ from its approximating Student's t distribution [26]. To test for differentially methylated regions (DMRs), we implemented Comb-p, a bioinformatics tool that groups individual CpGs by genomic location and proximity [27]. We used the Fisher-exact P values as input along with the genomic annotation. For the argument, we used a minimum distance of 500 base-pairs and required a P-value of 10^{-3} to start a region. For each CpG site found within a DMR, we report the ACE and the univariate Fisher-exact paired P-values (and asymptotic P values for comparison). All analyses were performed using the R statistical software.

Results

Demographic characteristics and exposure randomization order among the 13 healthy volunteers are shown in Table 1.

In epigenome-wide analyses, 80 CpGs achieved a minimum possible observable P-value ($2/2^{13}$ or $P = 2.44 \times 10^{-4}$) calculated from the experimental design and shown in Supplementary Table S1. A Manhattan plot of Fisher's exact P values on the logarithmic scale (y-axis) mapped to each chromosome (x-axis) is displayed in Fig. 1. The volcano plot, displaying the Fisher's exact P values on the logarithmic scale (y-axis) against the average causal effect (ACE) for each CpG (x-axis), is shown in Fig. 2. Mapping of the individual sites, genes, and effect sizes is displayed in a circular genomic map in Fig. 3. Of the 80 CpGs with the minimum observable P-value, 62 CpGs (77.5%) show increased DNA methylation with DE exposure relative to CA exposure. Among sites achieving the minimum Fisher-exact P-value [P-value$_{min}$ or $P < (2/2^{13})$], the largest ACE was observed at cg12413579 (ACE = 6.07%), which annotated to the Proteasome Subunit Beta 2 (*PSMB2*) gene. When comparing Fisher-exact P values to asymptotic methods, we observed that 28 CpGs succeed the P-value$_{min}$. Annotated results for the 80 differentially methylated sites based on Fisher-exact tests are available in the Supplementary Table S1. Manhattan, volcano, and quantile–quantile plots of asymptotic paired t-test P values are shown in Supplementary Figs S1–S3, respectively.

In regional DNA methylation analyses implemented with Comb-p [27] and based on the Fisher-exact P values, we observed two differentially methylated regions (DMRs). One of the regions was annotated to chromosome 5 (chr5:126 409 062–126 409 311) and covers seven CpGs that mapped to a CpG island on the chromosome 5 open reading frame 63 (*C5orf63*; clone name: *FLJ44606*) gene. Two of these seven sites achieved P-value$_{min}$ (cg10812634 and cg15405432). All sites within this region showed increased DNA methylation after DE exposure, with ACEs ranging from 2.7% (cg21113900) to 6.0% (cg10812634), and five of the seven sites were annotated to the transcription start site. The second region was found in chromosome 15 (chr15: 91 473 292–91 473 570) and covers five CpGs, with four sites annotated to a CpG island and one to the North Shore region of the unc-45 myosin chaperone A (*UNC45A*) gene. All five CpGs within this DMR showed increased DNA methylation, with ACEs ranging from 1.5% (cg08267442) to 4.0% (cg08551047) and two that
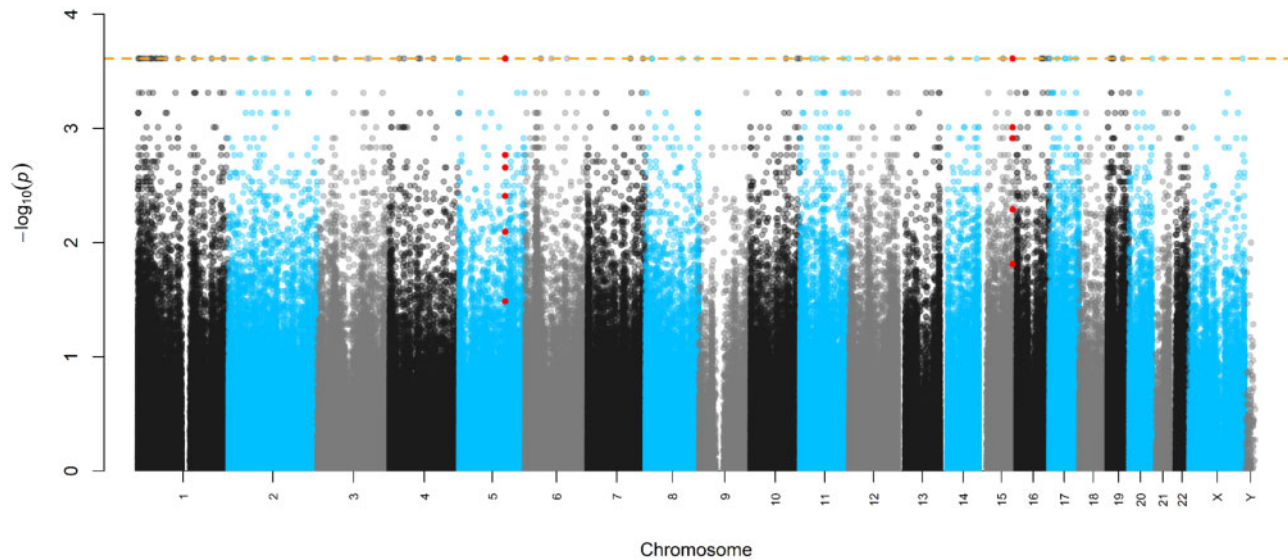
Figure 1: Manhattan plot of exact *P* values and chromosome position. The dashed orange line is the minimum possible *P*-value. CpGs annotated to the DMRs of chr5; *C5orf63* and chr15: *UNC45A* are colored in red.
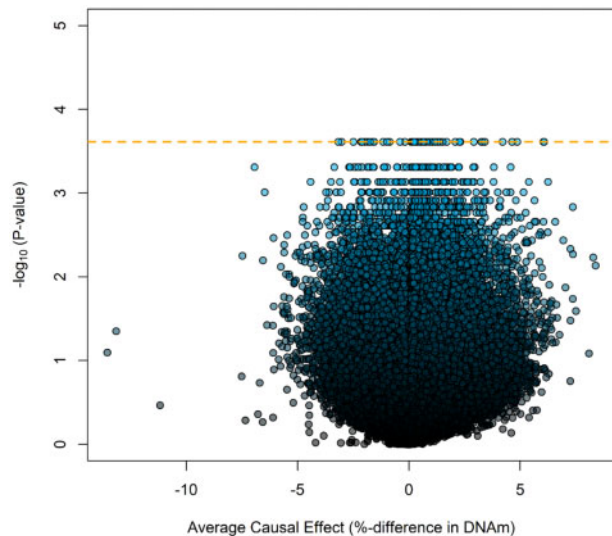

Figure 2: Volcano plot showing change in DNA methylation in bronchial cells against −log10 of exact *P*-values. The dashed orange line is the minimum possible *P*-value from an exact test.

were annotated to the transcription start site. Individual CpGs of DMRs with their ACEs, genomic annotation, and Fisher-exact *P* values are shown in Table 2.

We performed a gene functional enrichment analysis using the online tool DAVID 6.8 to investigate biological relationships among the genes mapped to the 80 CpGs shown to achieve *P*-value$_{min}$ [28]. The pathways that have an unadjusted *P*-value < 0.05 are displayed in the Supplementary Table S2. Although none of the pathways achieved statistical significance after multiple testing corrections, the two pathways with the highest enrichment scores include those for epidermal growth factor (EGF) and proteases. Finally, Supplementary Table S3 compares the effect sizes and *P* values of marginally significant CpGs (*P* < 0.05) that were found to be differentially methylated in two other experimental human exposure studies [29, 30] with the effect sizes and *P* values observed in this study.

Discussion

DE emissions from engines around the world contribute to outdoor air pollution that, when inhaled, increases risk for the development and exacerbation of pulmonary and cardiovascular diseases [31, 32]. While epidemiology studies demonstrate significant associations between DE exposure and disease incidence and severity, there is a lack of understanding of pollution-related epigenetic changes which may be involved in mediating or attenuating pathological development. In an epigenome-wide experiment of target bronchial epithelial cells in human subjects exposed to DE, we observed differential DNA methylation at 80 individual CpGs and increased DNA methylation of two regions of the *FLJ44606* and *UNC45A* genes. These results contribute to the growing literature aiming to elucidate the molecular underpinnings of how environmental pollution exposure can affect the epigenome of human bronchial epithelial cells [33, 34].

The *FLJ44606* gene encodes for a glutaredoxin-like protein YDR286C homolog. Glutaredoxins are thioltransferases that function as electron carriers in the glutathione-mediated synthesis of DNA nucleotides by ribonucleotide reductase [35]. An EWAS of Korean adult smokers and nonsmokers who had chronic obstructive pulmonary disease found a DMR of *FLJ44606* (chr5: 126 408 756–126 409 553) [36], which is in line with our data, showing that airway contaminants might target the *FLJ44606* gene.

The *UNC45A* (Unc-45 Myosin Chaperone A) gene encodes for a regulating component of the heat shock 90 (HSP90) chaperone that is believed to play a role in cell proliferation, the accumulation of myosin during muscle cell development, and the assembly of the progesterone receptor [37]. This protein contributes to tumorigenesis by regulating cancer cell proliferation [38]. In addition, biallelic loss-of-function mutations in this gene cause the development of osteo-oto-hepato-enteric syndrome, which includes the clinical features of cholestasis, congenital diarrhea, impaired hearing, and bone fragility [39]. Therefore, the protein encoded by *UNC45A* may have important physiological functions in several organ systems. An epigenetic study [40] found differential methylation of the *UNC45A* gene in patients

Figure 3: Circular map of genomic distribution among differentially methylated CpGs. From the outmost ring inward, the map shows gene names, chromosome number, position within the chromosome, and effect sizes among Average Causal Effects (below the gray line is a negative effect size and above the line is a positive effect size).

diagnosed with adrenoleukodystrophy, which is a neurodegenerative disorder characterized by dysregulated long-chain fatty acid metabolism—a pathologic process associated with exposure to traffic-related air pollutants [41]. We found increased DNA methylation of the UNC45A gene associated with DE exposure but in bronchial cells. In addition, DNA methylation of this gene in whole blood samples has been associated with FEV$_1$/FVC, a marker of lung function, among never smokers [42].

The gene functional enrichment analysis we performed for the DMRs showed the highest enrichment scores for EGF and proteases. Both are implicated in several acute and chronic respiratory diseases. EGF stimulates EGF receptors (EGFR) expressed on alveolar and airway epithelial cells to regulate

epithelial barrier function in the context of repair and injury [43]. Overexpression or continuous activation of EGFR due to mutations or environmental insults can lead to increased fibrotic processes, hyperproliferation of epithelial cells, increased epithelial permeability, and excessive mucin production. The downstream effects include higher risks for the development of acute lung injury, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, non-small cell lung cancer, and other lung conditions [44–48]. Proteolytic enzymes, such as metalloproteases produced by neutrophils and alveolar macrophages, have important physiological roles in tissue remodeling and repair in the lungs and airways [49]. However, dysregulation of proteases mediates the development of acute lung injury and

Table 2: Regional DNA methylation analyses of controlled human exposures to diesel exhaust utilizing the exact P values

Genomic coordinates	Gene	Software P-value	CpG sites	Position	Gene group	Relationship to CpG Island	Mean %-DNAm diesel exhaust (DE)	Mean %-DNAm clean air (CA)	Mean causal effect (ACE)	Exact P-value
chr5: 126 409 062– 126 409 311	C5orf63 (Clone name; FLJ44606)	1.44×10^{-4}	cg18710053	126409061	5'UTR	Island	24.49%	19.21%	5.28%	3.91×10^{-3}
			cg27097438	126409153	First Exon	Island	11.27%	7.62%	3.65%	8.06×10^{-3}
			cg22905511	126409198	TSS200	Island	14.89%	9.64%	5.25%	1.71×10^{-3}
			cg10812634	126409211	TSS200	Island	16.25%	10.21%	6.04%	$\mathbf{2.44 \times 10^{-4}}$
			cg02304092	126409227	TSS200	Island	15.67%	10.03%	5.64%	2.20×10^{-3}
			cg21113900	126409308	TSS200	Island	24.84%	22.10%	2.74%	3.27×10^{-2}
			cg15405432	126409310	TSS200	Island	10.74%	7.34%	3.41%	$\mathbf{2.44 \times 10^{-4}}$
chr15: 91 473 292– 91 473 570	UNC45A	1.71×10^{-7}	cg03291024	91473291	TSS200	North Shore	6.45%	4.39%	2.06%	1.22×10^{-3}
			cg08267442	91473386	TSS200	Island	6.99%	5.54%	1.45%	1.54×10^{-2}
			cg01351822	91473475	5'UTR	Island	6.17%	3.15%	3.03%	9.77×10^{-4}
			cg16414568	91473511	5'UTR	Island	7.46%	4.55%	2.91%	$\mathbf{2.44 \times 10^{-4}}$
			cg08551047	91473569	5'UTR	Island	18.60%	14.57%	4.04%	5.13×10^{-3}

Minimum P values are given in bold.

chronic inflammatory lung conditions [50]. Our findings suggest that the biological mechanisms explaining increased risk for lung disease associated with exposure to DE could be linked to methylation of genes that regulate EGF and proteases, but further research is needed.

None of the differential methylation, we found in 80 CpG sites in our participants matched CpG sites in studies examining changes in DNA methylation of blood mononuclear cells in nonsmoking patients with asthma exposed to DE [29] and in bronchial epithelial cells in humans exposed to DE and allergens [30]. However, some of the significant CpG sites that were differentially methylated in both these studies achieved a Fisher-exact P-value < 0.05 in our study. The direction of the effects—decreased methylation—for the CpGs in our study is the same as in the Clifford et al. study, which also examined bronchial cells. Regarding the epigenetic alterations in peripheral blood mononuclear cells in the DE study of Jiang et al., we found overlap in four CpG sites, three of which show the opposite direction of associations. Therefore, differential methylation of certain sites following DE exposure likely varies across tissues, but we were able to replicate findings from six previously reported sites in target bronchial tissue.

The level of DE exposure used in this trial, 300 μg/m^3 for 2 h, could be classified in the moderate range. For example, levels can range between 154–1600 μg/m^3 for miners, 121 μg/m^3 for construction workers, and 10–35 μg/m^3 for taxi drivers [51]. However, the composition of DE might play a significant role in the pathogenesis of its effects.

Our study has certain limitations. There is a small sample size with little variation in demographics. This limits the generalizability of our results and the power with which we can statistically detect effects; future studies should include more participants from diverse backgrounds. However, this was a randomized controlled exposure experiment, considered the gold standard to infer causality. The third exposure to O$_3$, not included in this investigation, could have influenced the effects of DE on the epigenome. However, the washout period of 13 days together with the randomization of the order of experimental condition should have minimized this possibility. In fact, none of the signals found for O$_3$ were observed in the findings for DE. Although the statistical power was limited given the number of tests across the epigenome, we found evidence that many sites reached the minimum Fisher-exact P-value. Additionally, the discovery of two robust DMRs with multiple CpG sites that have consistent direction of effects in epigenetically relevant CpG islands further strengthens the evidence for a causal effect of short-term DE exposure on the epigenome. We also did not have access to genetic information from study participants, so we did not exclude sites that might be associated with SNPs; therefore, the possibility of our findings to reflect genetic influence on methylation cannot be excluded. The collection of target bronchial cells is a major strength, as all observational studies have examined leukocyte DNA methylation, and our design provided a unique opportunity to study target tissue.

Conclusions

DE is a significant component of outdoor air pollution, and short-term exposure to DE alters bronchial epithelial cell DNA methylation in a randomized, controlled exposure study of healthy adults. Findings of differential methylation associated with the *FLJ44606* and *UNC45A* genes contribute to our knowledge of the epigenetic alterations that may precipitate the known adverse pulmonary and systemic effects of DE exposure. Functional studies on the target genes and sites may provide further evidence on the molecular impact of DE exposure on human health.

Supplementary data

Supplementary data are available at *EnvEpig* online.

Data Availability

The datasets used and/or analyzed during the current study are available from the study team on reasonable request.

Funding

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