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Long-term Air Pollution Exposure, Genome-wide DNA Methylation and Lung Function in the LifeLines Cohort Study

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Background: Long-term air pollution exposure is negatively associated with lung function, yet the mechanisms underlying this association are not fully clear. Differential DNA methylation may explain this association.

Objectives: Our main aim was to study the association between long-term air pollution exposure and DNA methylation.

Methods: We performed a genome-wide methylation study using robust linear regression models in 1,017 subjects from the LifeLines cohort study to analyze the association between exposure to nitrogen dioxide (NO_2) and particulate matter ($PM_{2.5}$, fine particulate matter with aerodynamic diameter $\leq$2.5 μm; PM_{10}, particulate matter with aerodynamic diameter $\leq$10 μm) and $PM_{2.5absorbance}$, indicator of elemental carbon content (estimated with land-use-regression models) with DNA methylation in whole blood (Illumina® HumanMethylation450K BeadChip). Replication of the top hits was attempted in two independent samples from the population-based Cooperative Health Research in the Region of Augsburg studies (KORA).

Results: Depending on the *p*-value threshold used, we found significant associations between NO_2 exposure and DNA methylation for seven CpG sites (Bonferroni corrected threshold $p < 1.19 \times 10^{-7}$) or for 4,980 CpG sites (False Discovery Rate <0.05). The top associated CpG site was annotated to the *PSMB9* gene (i.e., cg04908668). None of the seven Bonferroni significant CpG-sites were significantly replicated in the two KORA-cohorts. No associations were found for PM exposure.

Conclusions: Long-term NO_2 exposure was genome-wide significantly associated with DNA methylation in the identification cohort but not in the replication cohort. Future studies are needed to further elucidate the potential mechanisms underlying NO_2-exposure–related respiratory disease. https://doi.org/10.1289/EHP2045

Introduction

Air pollution is a major concern in public health. Long-term air pollution exposure has been consistently associated with lower function in adults (Ackermann-Liebrich et al. 1997; Adam et al. 2015; Forbes et al. 2009), children (Barone-Adesi et al. 2015; Gehring et al. 2013; Urman et al. 2014) and in those with preexisting lung disease (Nitschke et al. 2016). In the LifeLines cohort study, a large representative sample of the north of Netherlands, a higher level of nitrogen dioxide (NO_2) and particulate matter (PM) exposure was associated with lower forced expiratory volume in 1 s (FEV_1) and even lower forced vital capacity (FVC), and as a consequence, a higher FEV_1/FVC ratio (De Jong et al. 2016).

Activation of oxidant and pro-inflammatory pathways is suggested to be a potential mechanism underlying the acute toxicity of NO_2 and PM (Lodovici and Bigagli 2011), yet the knowledge on exact mechanisms underlying the effect of long-term air pollution exposure on health is inconclusive. Although clear evidence exists for the association between genetics and lung function (Hobbs et al. 2017), the genetic background cannot entirely explain the phenotypic variability. Emerging evidence suggests that apart from specific genetic variants, epigenetic alterations in response to environmental exposure is an important determinant of respiratory health (Boezen 2009). DNA methylation, currently the most frequently studied epigenetic mechanism, occurs by the binding of a methyl group to a cytosine-guanine dinucleotide (CpG site) (Griffiths et al. 2011). Changes in DNA methylation may be induced by exposure to air pollution and may alter the gene expression profile. This change in DNA methylation is one plausible mechanism potentially mediating the adverse health effects of air pollution (Holloway et al. 2012). Differential blood DNA methylation in response to air pollution exposure has been reported in environmental (Bind et al. 2014; De Prins et al. 2013), occupational (Sanchez-Guerra et al. 2015; Tarantini et al. 2009), and experimental settings (Ding et al. 2016). The strongest epigenetic signals identified in response to air pollution exposure were found in candidate genes for inflammatory pathways (*F3, ICAM-1, TLR-2*, *IFN-γ, IL-6*) (Bind et al. 2014), detoxification metabolism system (*GST*) (Madrigano et al. 2011), lung function (*TLR2, GCR*) (Lepeule et al. 2014), lung cancer (*SATα*, *NBL2*) (Hou et al. 2014), and biological aging (Ward-Caviness et al. 2016).

Despite these findings, no study to date has examined whether the association between long-term air pollution exposure and

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lung function is mediated by DNA methylation (Tarantini et al. 2009). In this study, we performed a genome-wide methylation study of the long-term air pollution exposure, i.e., NO_2 and PM in adults from the LifeLines cohort study. We further investigated to what extent the significant air pollution-associated DNA methylation sites mediated the association between air pollution and lung function.

Methods

Study Population and Design

This study included subjects enrolled in the LifeLines cohort study. LifeLines is a large Dutch population-based cohort study designed to investigate chronic diseases and healthy aging (Scholtens et al. 2015). Detailed information about LifeLines can be obtained at the official website (http://www.lifelines.net). The LifeLines cohort study was approved by the Medical Ethical Committee of the University Medical Center Groningen, Groningen, Netherlands. All participants provided written informed consent.

A subgroup of 1,656 subjects of the LifeLines cohort was selected based on having complete data on lung function and specific environmental exposures as well as on covariates used in the analysis: sex, age, height, and smoking history.

Air Pollution Exposure Assessment

At the baseline visit (2007–2013), the home addresses of all LifeLines individuals were geocoded and GIS (geographic information system)-derived information on distance to the nearest road, traffic intensity, built-up land, population density, and altitude was acquired. The annual average exposure to NO_2, PM_{10} (PM with aerodynamic diameter less than 10 μm), $PM_{2.5}$ (particles with aerodynamic diameter less than 2.5 μm) and $PM_{2.5absorbance}$ (indicator of elemental carbon content) (Cyrys et al. 2003) was then estimated using land-use regression models, developed in the ESCAPE study. In these models, the GIS-derived information was combined with annual air pollution concentrations from an intensive monitoring campaign in the ESCAPE study (Beelen et al. 2013; Eeftens et al. 2012; Zijlema et al. 2016).

Lung Function Assessment

Lung function was measured with spirometry according to standard operating procedures using the Wellch Allyn SpiroPerfect device (Wellch Allyn version 1.6.0.489, personal computer-based SpiroPerfect™ with CardioPerfect® Workstation software). FEV_1, FVC, FEV_1/FVC, and forced expiratory flow between 25–75% of the FVC ($FEF_{25–75}$) were used as lung-function outcomes.

Covariate Assessment

Age was calculated based on the date of birth as registered in the municipal registries and the date of the baseline visit. Body mass index (BMI) is calculated as weight divided by height squared, as measured during the baseline visit using standardized procedures. Smoking status and cumulative smoking exposure (pack-years of cigarettes smoked) were assessed using the standardized European Community Respiratory Health Survey (ECRHS) questionnaire (Burney et al. 1994). Current smoking was defined as smoking in the last month, and only current smokers with a smoking history greater than five pack-years were included. Never-smokers were defined as having a smoking history of zero pack-years. To optimize the smoking exposure contrast, ex-smokers were not included in this study.

Genome-wide DNA Methylation Assessment

Genome-wide DNA methylation levels were assessed from whole blood of 1,656 subjects using standard methods. Briefly, blood samples were bisulfite-treated (EZ-96 DNA Methylation™ Kit; Zymo Research Corp.) and subsequently subjected to whole-genome amplification. DNA methylation level for each CpG site was measured using the Illumina Infinium® Human Methylation 450K array (Illumina, Inc.) and expressed quantitatively as β-value. β-Values represent the ratio of the fluorescent signal intensity measured by methylated and unmethylated probes and range from 0 (all copies of the CpG site in the sample are unmethylated) to 1 (all copies of the CpG site in the sample are methylated). Quality control (QC) included removal of samples with probes with a detection p-value >0.01 in <99% of probes, samples with incorrect sex or SNP prediction, as well as probes with a detection p-value >0.01, sex chromosome probes, probes measuring SNPs, probes where the CpG itself or the single base extension (SBE) site is an SNP, and cross-reactive probes. A total of 420,938 CpG sites passed the QC filtering criteria. The data were normalized using DASEN implemented in the wateRmelon package in R (R Core Team) (Pidsley et al. 2013).

Replication Analysis

Replication of the top CpG sites associated with air pollution exposure was attempted in two independent samples from the population-based Cooperative Health Research in the Region of Augsburg studies [Kooperative Gesundheitsforschung in der Region Augsburg, Germany (KORA F3 and KORA F4)]. The KORA F3 examinations took place from 2004 to 2005 (Aulchenko et al. 2009; Wichmann et al. 2005), and KORA F4 examinations took place from 2006 to 2008 (Rückert et al. 2011). For both examinations, health surveys were administered, and biospecimens were collected by trained personnel per published methodologies. Informed consent was provided by all participating individuals. All KORA studies were approved by the ethics committee of the Bavarian Medical Association in Munich, Germany. The KORA F3 and KORA F4 samples used for this replication study were nonoverlapping. Air pollution at the residential address was estimated using land-use regression models as developed in the ESCAPE study (Pitchika et al. 2017). DNA methylation was assessed identically for the KORA F3 and KORA F4 samples. Genome-wide DNA methylation measurement at 485,577 genomic sites was performed using the Infinium® HumanMethylation450K BeadChip (Illumina, Inc.) and expressed quantitatively as β-value. The laboratory process has been described previously (Zeilinger et al. 2013). To preprocess the DNA methylation data, first, 65 probes that represent SNPs were excluded. Next, background correction using minfi, version 1.6.0 (Aryee et al. 2014) was performed, and signals represented by fewer than three functional beads were removed. Data were normalized using quantile normalization (QN) on the raw signal intensities (Lehne et al. 2015). QN was performed on six stratified probe categories based on probe type and color channel (Bibikova et al. 2011) using the R package limma (version 3.16.5; R Core Team). Differences in the signal intensities from Infinium I vs. Infinium II probes designs were corrected using beta-mixture quantile normalization (BMIQ) (Teschendorff et al. 2012) via the R package wateRmelon, version 1.0.3 (R Core Team) (Pidsley et al. 2013). White blood cell (i.e., granulocytes, monocytes, B cells, CD4+ T cells, CD8+ T cells, and natural killer cells) proportions were estimated using the Houseman method (Houseman et al. 2012). To keep in concert with the discovery analyses, individuals with a detection p-value >0.05

for >1% of the probes were removed. After quality control, 451 samples were retained in KORA F3 and 1,424 in KORA F4.

Statistical Analyses

Statistical analyses were performed using the SPSS statistics software version 23.0 (IBM) and R software version 3.2.4 revised (R Foundation). Robust linear regression models were used to test the cross-sectional association between air pollution (NO_2, PM_{10}, $PM_{2.5}$, and $PM_{2.5absorbance}$) exposure as a predictor and genome-wide DNA methylation levels as a response. The models were adjusted for sex, age, BMI, current smoking, pack-years, and covariates expected to influence the DNA methylation levels (technical covariates and blood cell composition). The potential technical bias was minimized using principal component analysis applied to the control probes included on the 450K chip (Lehne et al. 2015). We included all PCs that explained >1% of the variance. This resulted in the inclusion of the first 7 PCs that together explained 95.5% of total variance. Additionally, the model was adjusted for the measured white blood cell counts (eosinophils, neutrophils, basophils, lymphocytes, and monocytes) to correct for the cellular heterogeneity of blood samples (Jaffe and Irizarry 2014). We used the Bonferroni corrected threshold p-value $<1.19\times10^{-7}$ (0.05/420,938) to correct for the number of CpG sites tested. Sites that passed this threshold were considered genome-wide significant and were investigated further in subsequent analyses. To investigate the sensitivity of the results of the analyses between air pollution exposure and methylation to the model specifications we conducted several sensitivity analyses to the top hits of our analyses (see Supplemental Material for details): *a*) exclusion of outliers in the DNA-methylation levels, *b*) additional adjustment for possible confounders (i.e., highest educational level, chronic obstructive pulmonary disease (COPD), asthma, use of respiratory medication), and *c*) stratification of the models by sex, BMI, and smoking. Replication of the top hits was attempted in two independent samples from the KORA study. In this replication analysis, associations were estimated using robust linear regression models and included the following covariates: sex, age, body mass index, current smoking, pack-years, estimated cell counts, and the first 20 PCs from the control probes to adjust for technical variation (Lehne et al. 2015). As in the discovery analysis, only, never, and current smokers were included in the analysis (ex-smokers were excluded). Significant replication is defined as a p-value <0.05 in at least one of the replication cohorts, and the direction of the effect should be the same in the discovery and both replication cohorts. In addition, using the software tool provided at https://129.125.135.180:8080/GeneNetwork/pathway.html, we conducted a pathway analysis in which we included all genes annotated to CpG sites with an FDR p-value <0.01, and we investigated the association between our top methylation-sites and gene expression by searching the tables provided at https://www.genenetwork.nl/biosqtlbrowser/.

Robust linear regression models adjusted for sex, age, height, BMI, sex*age interaction, sex*height interaction, current smoking, and pack-years were used to analyze the cross-sectional association between air pollution exposure and lung function levels, as measured by: FEV_1, FVC, FEV_1/FVC, and $FEF_{25–75}$. Two-sided p-values <0.05 were considered statistically significant.

The potential mediation by significant air pollution-associated methylation sites was assessed using mediation analysis. By applying the bootstrapping method in the "mediation" package in R (version 4.4.6; R Core Team) (Hayes 2009), we verified whether the total effect of a specific air pollutant on a lung function outcome was mediated by DNA methylation at the significant CpG sites. To test this mediation effect, two models were applied, and their estimates were used as input for the mediate function (Figure 1). The first model, the mediator model, assessed the effect of air pollution exposure on DNA methylation (association A) and, the second model, the outcome model, assessed the combined effect of air pollution exposure and the mediator (DNA methylation) on a lung function outcome (associations B and C). A total of 1,000 bootstraps were run to estimate the confidence intervals (CIs) (Mayer et al. 2014). Significant mediation by DNA methylation was considered present when the p-value of the average mediation effect (AME) was <0.05. A p-value between 0.05 and 0.10 was considered as borderline significant.

Results

Descriptive Statistics

In total, 1,622 subjects passed the quality control of the DNA methylation assessment, and 1,017 subjects had complete data on air pollution exposure, DNA methylation levels, and all included covariates. Table 1 summarizes the baseline characteristics of the 1,017 subjects with complete data and 605 subjects with incomplete data. The mean age of the complete subjects was 47.3 y, 58% were male, and 56% were never smokers. In current smokers, the mean cumulative smoking exposure was 20.5 pack-years. Mean levels of lung function measured at the baseline visit were: FEV_1 3.5L (98% of predicted normal values), FVC 3.5L (111% of predicted), FEV_1/FVC 73.4%, and $FEF_{25–75}$ 2.8 L/s (70% of predicted) (Quanjer et al. 1993). No significant differences were found between the incomplete and complete subjects. The annual average estimated concentration (range) of exposure was $16.3\,(9.4-32.8)\,\mu g/m^3$ for NO_2; $24.1\,(23.7-27.6)\,\mu g/m^3$ for PM_{10}; $15.5\,(15.1-18.2)\,\mu g/m^3$ for $PM_{2.5}$, and $0.9\,(0.8-1.6)\times10^{-5}m^{-1}$ for $PM_{2.5absorbance}$. The correlation (Pearson correlation coefficient) among the annual average concentration of pollutants is presented in Supplemental Material (Table S1).

Association between Air Pollution Exposure and Genome-Wide DNA Methylation

NO_2 exposure was genome-wide significantly associated with differential DNA methylation at seven CpG sites (mapped to seven different genes) at the Bonferroni corrected threshold p-value $<1.19\times10^{-7}$ and with 4,980 CpGs at the False Discovery Rate (FDR) p-value <0.05 (Table 2, see also Excel Table S1 and Figure S1). Among these top signals, three CpG sites

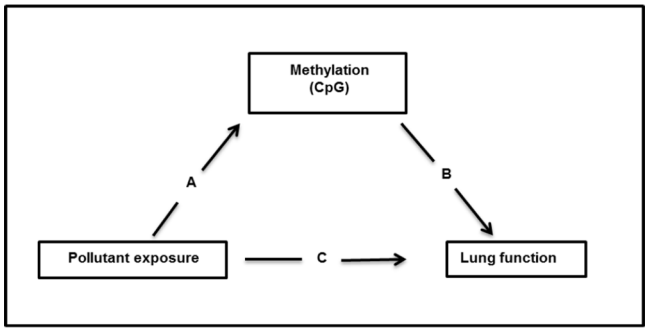

Figure 1. Model showing the associations tested in the mediation analysis. Association A: association between air pollution and DNA methylation; association B: association between DNA methylation and lung function; association C: association between air pollution and lung function with adjustment for DNA methylation.

Table 1. Characteristics of subjects with incomplete or complete data from the LifeLines Cohort Study.

Characteristic	Incomplete data	Complete data	Difference *p*-Value
N (%)	605 (37.3)	1,017 (62.7)	
Age (y)	46.8 ± 10.6	47.3 ± 11.0	0.38
Height (cm)	176.4 ± 9.1	177.2 ± 9.1	0.08
BMI (kg/cm^2)	26.4 ± 4.1	26.0 ± 3.8	0.07
Male	341 (56.4)	587 (57.7)	0.59
Smoking status			
Never smoking	343 (56.7)	574 (56.4)	0.92
Current smoking[a]	262 (43.3)	443 (43.6)	
Cumulative smoking[b]	22.0 ± 11.4	20.5 ± 11.8	0.09
Lung function			
FEV_1 (L)	3.4 ± 0.9	3.5 ± 0.9	0.07
FVC (L)	4.7 ± 1.1	4.7 ± 1.1	0.17
FEV_1/FVC (%)	72.8 ± 8.8	73.4 ± 8.6	0.18
$FEF_{25–75}$ (L/s)	2.7 ± 1.3	2.8 ± 1.3	0.08
Air pollution			IQR
NO_2 (μg/m^3)	NA	16.3 ± 3.2	4.66
PM_{10} (μg/m^3)	NA	24.1 ± 0.5	0.58
$PM_{2.5}$ (μg/m^3)	NA	15.5 ± 0.2	0.13
$PM_{2.5absorbance}$ (10^{-5}/m)	NA	0.9 ± 0.1	0.13

Note: Data are presented as mean ± standard deviation (SD) for continuous variables or *N* (%) for categorical variables; BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FCV, forced vital capacity; FEV_1/FVC, forced expiratory volume in 1 sec to forced vital capacity ratio; $FEF_{25–75}$, forced expiratory flow between 25–75% of the FVC; NO_2, nitrogen dioxide; PM_{10}, particles with aerodynamic diameter <10 μm; $PM_{2.5}$, particles with aerodynamic diameter <2.5 μm; $PM_{2.5absorbance}$, indicator of elemental carbon content.
[a]Current smoking is defined as smoking in the last month and only current smokers with a smoking history greater than five pack-years were included.
[b]Cumulative smoking defined by pack-years of cigarettes smoked in current smokers.

showed a negative association with NO_2 exposure: cg04908668 (*PSMB9*, chr6), cg00344801 (*TTC38*, chr22), and cg02234653 (*AP1S3*, chr2); four showed a positive association: cg14938677 (*ARF5*, chr7), cg18379295 (*GNG2*, chr14), cg25769469 (*PTCD2*, chr5), and cg08500171 (*BAT2*, chr6). The results of the sensitivity analyses on these 7 CpG sites are presented in Tables S2, S3, and S4. Importantly, after removal of outliers in the methylation values 1 CpG (i.e., cg02234653) was no longer significant at the Bonferroni corrected threshold although the effect estimate remained similar (Table S2). None of the genome-wide significant CpG sites associated with NO_2 exposure was successfully replicated either in the KORA F3 or in the KORA F4 cohort (Tables 3 and 4). The results of the pathway analyses are presented in Excel Tables S2 and S3. A look-up of these 7 CpG sites in the eQTM table provided at https://www.genenetwork.nl/biosqtlbrowser/ showed that 2 CpGs were associated with gene expression of 3 genes [i.e., cg04908668 was associated with lower expression of *Proteasome Subunit Beta 9* (*PSMB9*) and of *Transporter 1, ATP Binding Cassette Subfamily B Member* (*TAP1*) genes, and cg00344801 was associated with higher expression of *Tetratricopeptide Repeat Domain 38* (*TTC38*)] (Table S5). PM_{10}, $PM_{2.5}$, and $PM_{2.5absorbance}$ exposures were not genome-wide significantly associated with DNA methylation, either at the Bonferroni corrected threshold or at the FDR-threshold (see Table S6 and Excel Table S4 and Table S7 for CpG sites associated with a *p*-value $<1\times10^{-5}$). Given that only NO_2 exposure was genome-wide significantly associated with DNA methylation, the subsequent mediation analyses were restricted to NO_2.

Table 3. Characteristics of the subjects included in the discovery and replication analyses.

Characteristic	Discovery cohort	Replication cohorts KORA F3	KORA F4
N	1,017	422	971
Age (y)	47.3 ± 11.0	53.2 ± 9.6	60.9 ± 8.8
BMI (kg/cm^2)	26.0 ± 3.8	27.1 ± 4.4	27.8 ± 4.6
Male	587 (57.7)	261 (51.2)	383 (39.4)
Smoking status			
Never smoking	574 (56.4)	205 (48.6)	721 (74.3)
Current smoking[a]	443 (43.6)	217 (51.4)	250 (25.7)
Cumulative smoking[b]	20.5 ± 11.8	26.2 ± 20.6	30.6 ± 24.4
Air pollution			
NO_2 (μg/m^3)	16.3 ± 3.2	18.3 ± 3.7	18.8 ± 3.9

Note: Data are presented as mean ± standard deviation (SD) for continuous variables or *N* (%) for categorical variables. BMI, body mass index; NO_2, nitrogen dioxide.
[a]Current smoking is defined as smoking in the last month and only current smokers with a smoking history greater than five pack-years were included.
[b]Cumulative smoking defined by pack-years of cigarettes smoked in current smokers.

Association between Air Pollution Exposure and Lung Function

Table 5 shows the association between NO_2 exposure and lung function levels (see Table S8 for the associations between all pollutants and lung function). NO_2 exposure was borderline significantly associated with FVC (B per 10 μg/m^3 NO_2 = −106.3, 95% CI = −219.1 − 6.6, p = 0.065) and with FEV_1/FVC (B = 1.5, 95% CI = −0.1 − 3.0, p = 0.060). FEV_1 was not significantly associated with NO_2 exposure, indicating that the positive association between NO_2 and FEV_1/FVC is driven by a stronger negative association with FVC than FEV_1. Given that NO_2 exposure was associated with FVC and with FEV_1/FVC, we examined the mediation by DNA methylation for FEV_1, FVC and FEV_1/FVC.

Mediation Analysis

Mediation analysis showed that one of the seven top CpG sites significantly mediated the association between NO_2 exposure and FVC (cg14938677), and 2 CpG sites significantly mediated the association between NO_2 and FEV_1/FVC (cg14938677 and cg18379295) (Table S9).

Table 2. Genome-wide differential DNA methylation associated with NO_2 exposure (per 10 μg/m^3) in the LifeLines Cohort Study (n = 1,017).

CpG site	B ± SE[a]	*p*-Value[b]	Chr	Bp position	Gene	Location in gene	Relation to island
cg04908668	−0.012 ± 0.002	7.94×10^{-9}	6	32823941	PSMB9	Body	S_Shore
cg14938677	0.023 ± 0.004	1.05×10^{-8}	7	127231698	ARF5	3'UTR	S_Shelf
cg00344801	−0.028 ± 0.005	2.38×10^{-8}	22	46685728	TTC38	Body	Island
cg18379295	0.020 ± 0.004	3.50×10^{-8}	14	52326155	GNG2	TSS1500	OpenSea
cg25769469	0.035 ± 0.006	3.69×10^{-8}	5	71643841	PTCD2	Body	OpenSea
cg02234653	−0.017 ± 0.003	4.07×10^{-8}	2	224625080	AP1S3	Body	OpenSea
cg08500171	0.023 ± 0.004	9.81×10^{-8}	6	31590674	BAT2	Body	S_Shore

[a]Effect estimates ± standard error (SE) of DNA methylation per 10-μg/m^3 increase in NO_2 concentration adjusted for sex, age, BMI, current smoking, pack-years, technical variance, and blood cell composition. Chr, chromosome.
[b]Bonferroni corrected threshold *p*-value $<1.19\times10^{-7}$.

Table 4. Replication analysis: robust linear regression analysis for the association between NO_2 and DNA methylation at the 7 genome-wide significant CpG sites in the KORA F3 and F4 cohort.

	Discovery cohort ($n = 1{,}017$)			KORA F3 ($n = 422$)			KORA F4 ($n = 971$)		
CpG	B	SE	*p*-Value	B	SE	*p*-Value	B	SE	*p*-Value
cg04908668	−0.012	0.002	7.94×10^{-9}	−0.002	0.002	0.260	0.000	0.002	0.446
cg14938677	0.023	0.004	1.05×10^{-8}	−0.008	0.003	0.013	0.001	0.003	0.394
cg00344801	−0.028	0.005	2.38×10^{-8}	−0.002	0.004	0.258	−0.001	0.003	0.421
cg18379295	0.020	0.004	3.50×10^{-8}	−0.004	0.004	0.147	−0.002	0.003	0.233
cg25769469	0.035	0.006	3.69×10^{-8}	0.003	0.006	0.292	−0.008	0.005	0.056
cg02234653	−0.017	0.003	4.07×10^{-8}	0.003	0.003	0.198	−0.002	0.002	0.149
cg08500171	0.023	0.004	9.81×10^{-8}	−0.003	0.004	0.202	−0.002	0.003	0.269

Note: Effect estimate per 10-μg/m^3 increase in NO_2 concentration. Robust linear regression analysis adjusted for sex, age, BMI, current smoking, pack-years, technical variance. and blood cell composition.

Discussion

We performed a cross-sectional genome-wide methylation study in blood to investigate whether long-term air pollution exposure is associated with DNA methylation in the LifeLines cohort study. We further investigated whether the association between air pollution exposure and lung function was mediated by DNA methylation. In our genome-wide methylation study, we identified differential DNA methylation at seven CpG sites to be genome-wide significantly associated with NO_2 exposure. After removal of outliers in the methylation values, six CpG sites remained significantly associated with NO_2 levels. Unfortunately, none of these associations could be significantly replicated in two independent cohorts. Further, higher levels of NO_2 exposure were borderline significantly associated with lower FVC and higher FEV_1/FVC levels. Finally, we found one out of seven CpG sites (cg14938677 in *ARF5*) was a significant mediator between NO_2 exposure and FVC, and two CpG sites (cg14938677 in *ARF5* and cg18379295 in *GNG2*) were significant mediators of the association between NO_2 exposure and FEV_1/FVC.

The top-significant CpG site (cg04908668) identified in our genome-wide methylation study on NO_2 maps to *PSMB9* (chromosome 6) and is associated with lower gene expression of *PSMB9* and *TAP1* (https://www.genenetwork.nl/biosqtlbrowser). *PSMB9* and *TAP1* are suggested to be involved in the pathophysiological mechanisms underlying COPD. Fujino et al. (2012) report both *PSMB9* and *TAP1* to be differentially expressed in alveolar epithelial type II cells isolated from COPD patients, in comparison with healthy subjects. The putative function of all genes identified in our study is presented in the Supplemental Material (Table S10). Interestingly, 2 of the 7 genome-wide significant CpG sites (i.e., cg14938677 and cg00344801) were also described by Joehanes et al. (2016) in relation to smoking habits, which might indicate they are general markers of inhaled particle exposure.

Table 5. Effect estimates of NO_2 exposure on lung function (FEV_1 , FVC, FEV_1/FVC and $FEF_{25–75}$) for 1,017 subjects enrolled in the LifeLines Cohort Study.

	NO_2	
Variable	B (95% CI)[a]	*p*-Value
FEV_1 (mL)[b]	2.4 (−94.8, 99.6)	0.962
FVC (mL)[b]	−106.3 (−219.1, 6.6)	0.065
FEV_1/FVC (%)[c]	1.5 (−0.1, 3.0)	0.06
$FEF_{25–75}$ (mL/s)[d]	128.5 (−69.8, 326.8)	0.204

[a]Effect estimates and 95% confidence interval (95% CI) for NO_2 are given per 10-μg/m^3 increase.
[b]For FEV_1 and FVC, robust regression analysis adjusted for sex, age, height, BMI, sex*age interaction, sex*height interaction, current-smoking, and pack-years.
[c]For FEV_1/FVC, robust regression analysis adjusted for sex, age, BMI, sex*age interaction, current-smoking, and pack-years.
[d]For $FEF_{25–75}$, robust regression analysis adjusted for sex, age, height, BMI, sex*age interaction, sex*height interaction, current-smoking, pack-years, and FVC.

To date, genome-wide DNA methylation analyses of NO_2 allowing a hypothesis-free assessment of epigenetic modifications are scarce. However, relevant evidence comes from a study in children by Gruzieva et al. (2017). In this epigenome-wide meta-analysis of methylation, prenatal NO_2 exposure was associated with differential DNA methylation of genes involved in mitochondria and antioxidant defense pathways. Interestingly, in our genome-wide methylation study on NO_2, we also identified a CpG (cg25769469) in *PTCD2* that is reported to be involved in the mitochondrial RNA metabolism.

The positive association between NO_2 exposure and FEV_1/FVC found in our study is in line with findings reported in a larger sample of the LifeLines cohort ($n = 51{,}855$ subjects) (De Jong et al. 2016). In this larger sample, NO_2 had a stronger negative association with FVC than with FEV_1 resulting in a higher FEV_1/FVC. In our current smaller sample, the lack of significant association between NO_2 and FEV_1 may be the result of low study power (due to smaller sample size and smaller air pollution ranges). FEV_1 and FVC are considered early indicators of chronic respiratory disease and predictors for cardiorespiratory mortality (Lee et al. 2011). Clinically, a reduced FVC along with FEV_1 within a normal range is indicative (but not specific) of restrictive ventilatory abnormalities (Pellegrino et al. 2005). A comparison with existing studies shows that this restrictive effect is not universally seen. For example, in the ESCAPE study, the negative association between NO_2 exposure and FEV_1 and FVC are of equal magnitude (Adam et al. 2015). Because the main parameter for the diagnosis of restriction is a low total lung capacity (TLC), further studies including TLC are warranted to better elucidate whether the observed ventilatory pattern associated with NO_2 exposure corresponds to a restrictive, obstructive or both types of disorders.

We tested mediation by DNA methylation to confirm our hypothesis that NO_2 exposure may affect lung function through effects on DNA methylation. Among the seven differentially methylated CpG sites, two showed suggestive evidence for mediation (cg14938677 in *ARF5* and cg18379295 in *GNG2*). *ARF5* is a member of the human ADP-ribosylation factor (ARF) gene family that encodes small guanine nucleotide binding proteins. These proteins activate the phospholipase D (PLD), a critical enzyme involved in various endothelial and epithelial cell functions, such as actin cytoskeleton, vesicle trafficking for secretion, and endocytosis and receptor signaling (Jenkins and Frohman 2005). A family member, PLA1, was found to be significantly increased in plasma membrane of NO_2-exposed pulmonary artery endothelial cells (Bhat et al. 1990; Sekharam et al. 1991). Furthermore, the redox regulation of bleomycin-induced PLD activation was reported to play a crucial role in the cytotoxicity underlying the idiopathic pulmonary fibrosis (Patel et al. 2011). The *GNG2* (G protein subunit gamma 2) gene belongs to the heterotrimeric G protein family that underlies important pathways involved in cell migration, proliferation, differentiation,

apoptosis, and responses to external signals (Olate and Allende 1991). Its distinct isoform subunits α, β, and γ are selectively expressed and enriched in different tissues including white blood cells and lung (Modarressi et al. 2000). To date, we do not know whether differential DNA methylation at this particular CpG site (cg18379295), located in the transcription start site of the gene, results in any functional variation in lung function. However, *GNG2* was reported to be involved in airway hyper-responsiveness and inflammation elicited by an antigen challenge in a rabbit model of asthma (Nino et al. 2012). Furthermore, upon activation by G protein-coupled receptors (GPCRs), both free Gα and Gβγ subunits regulate important signaling pathways like the MAPK kinase cascade. This MAPK kinase cascade is involved in various immune and inflammatory cell functions and is a plausible mechanism linking air pollution exposure and respiratory and cardiovascular outcomes (Carmona et al. 2014).

A large number of studies have linked short- and mid-term PM exposure to global and gene-specific DNA methylation (Baccarelli et al. 2009; Bellavia et al. 2013; Chen et al. 2016; Peng et al. 2016; Wang et al. 2016). A genome-wide meta-analysis of DNA methylation and $PM_{2.5}$ identified twelve genes regulating pathways involved in tumor development, inflammatory stimuli, pulmonary disorders and glucose metabolism (Panni et al. 2016). However, few studies have examined this association in the context of a long-term exposure window (Chi et al. 2016; Ward-Caviness et al. 2016). Although we found no genome-wide significant effect of PM exposure (considering all different size fractions) on DNA methylation, many CpG sites had suggestive effects, especially in response to $PM_{2.5}$ (Table S8). Possibly, the relatively small range of PM levels and consequently a modest exposure contrast in LifeLines cohort may explain this lack of association. Future genome-wide methylation studies conducted in cohorts with a broader range of PM exposure is needed to clarify this association.

Our study is the largest genome-wide methylation study of air pollution exposure in adults, and the first study to assess the mediation effect of DNA methylation in the association between air pollution and the FEV_1/FVC ratio. However, this study has some limitations. We used the individual's home address as basis for the air pollution exposure estimates, ignoring the fact that a person could spend time in another environment (e.g., while traveling or working), which might lead to some degree of exposure misclassification at the individual level (Sunyer 2009). Interestingly, this exposure misclassification may lead to overestimation of the mediation effect (Valeri et al. 2017) when the methylation levels at our identified CpG sites are better biomarkers of personal NO_2 exposure than the estimated NO_2 exposure using land-use-regression models. The results of the mediation analyses should thus be interpreted with caution. In addition, because our study was cross-sectional in design, the inference of causality from these measures could be questionable.

We also recognize that ambient air pollution is a complex mixture and the effects attributed to some specific component might be influenced by the underlying toxicity of the full mixture of all pollutants. In this study, we estimated the association between various pollutants and DNA methylation, but only found genome-wide significant associations with NO_2. The moderate to high correlation between NO_2 and other pollutants prohibits the use of multipollutant models, and thus we cannot completely disentangle the independent pollutant effect. However, the top CpG sites differ for the different pollutants, indicating that each pollutant may have its own specific methylation target sites.

Another potential limitation of this study is the use of DNA methylation in blood samples when the outcome of interest is lung function. To what extent these epigenetic changes that we observe in peripheral blood cells reflect changes in DNA methylation in target tissues like the lung merits further investigation. Finally, the identified associations between NO_2 exposure and DNA methylation did not replicate in two independent cohorts from the German KORA study. This lack of replication could be explained by the differences in age, gender, BMI, and smoking habits between the cohorts (see Table 3), and therefore more replication studies should be performed to validate these findings.

Conclusions

In the largest genome-wide methylation study to date, long-term NO_2 exposure was associated with differential DNA methylation in blood in 1,017 subjects from the LifeLines cohort study. Among the significant NO_2-associated DNA methylation sites, 2 CpGs can be considered potential mediators of the association between NO_2 exposure and lung function. In this perspective, replication of these findings in other cohorts is necessary to elucidate the suggested role of epigenetic variability in the pathogenesis of NO_2-exposure-related respiratory disease.

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