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### **Supplemental Material**

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

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**Additional File: Excel document.**

## References

## **Supplemental methods:**

### **Sensitivity analyses**

To investigate the sensitivity of the results of the analyses between air pollution exposure and methylation to the model specifications we applied the following sensitivity analyses to the 7 CpG sites that were genome-wide significantly associated with NO<sub>2</sub> in our analyses:

#### **1. Exclusion of outliers in the DNA methylation levels**

The analyses on the 7 CpG sites that were genome-wide significantly associated with NO<sub>2</sub> were repeated with exclusion of outliers in the methylation levels. An outlier is defined as an observation being 3 times the interquartile range (IQR) lower than the 25<sup>th</sup> percentile or higher than the 75<sup>th</sup> percentile.

#### **2. Additional adjustment of the models for possible confounders**

The analyses on the 7 CpG sites that were genome-wide significantly associated with NO<sub>2</sub> were repeated with additional adjustment for the following possible confounders:

- a. Highest educational level: defined as low (no education, primary education, or lower vocational education), intermediate (secondary education or intermediate vocational education), high (higher vocational education or university)
- b. COPD: defined as FEV<sub>1</sub>/FVC<0.7
- c. Asthma: defined as self-reported doctor's diagnosed asthma
- d. Use of any respiratory medication: defined as any of beta-2 agonists, inhaled corticosteroids, anti-cholinergics, cromoglycates, theophyllins, or leukotriene-modifiers

### 3. Models stratified by sex, BMI, and smoking

The analyses on the 7 CpG sites that were genome-wide significantly associated with NO<sub>2</sub> were stratified for:

- a. Sex: male and female
- b. BMI: normal weight (BMI<25 kg/m<sup>2</sup>) and overweight (BMI≥25 kg/m<sup>2</sup>)
- c. Smoking: never smokers and current smokers

**Table S1.** Pearson correlation coefficient between the annual average concentrations of air pollutants (NO<sub>2</sub>, PM<sub>10</sub>, PM<sub>2.5</sub> and PM<sub>2.5</sub> absorbance) measured in the LifeLines cohort study (n=1,017).

<b>Pollutant</b>	PM <sub>10</sub>	PM <sub>2.5</sub>	PM <sub>2.5</sub> absorbance <sup>d</sup>
NO <sub>2</sub> <sup>a</sup>	0.7	0.4	0.8
PM <sub>10</sub> <sup>b</sup>		0.7	0.9
PM <sub>2.5</sub> <sup>c</sup>			0.7

<sup>a</sup> NO<sub>2</sub>: nitrogen dioxide

<sup>b</sup> PM<sub>10</sub>: particles with aerodynamic diameter less than 10µm

<sup>c</sup> PM<sub>2.5</sub>: particles with aerodynamic diameter less than 5µm

<sup>d</sup> PM<sub>2.5</sub> absorbance: indicator of elemental carbon content

**Table S2.** Sensitivity analysis: Robust linear regression analysis for the association between NO<sub>2</sub> and DNA methylation at the 7 genome-wide significant CpG sites before and after exclusion of DNA methylation outliers (based on 3\*IQR below the 25<sup>th</sup> percentile or above the 75<sup>th</sup> percentile).

CpG	Test	Original model (n= 1,017)			# of outliers	Without outliers		
		B	SE	p-value		B	SE	p-value
cg04908668	NO <sub>2</sub>	-0.012	0.002	7.94E-09	6	-0.011	0.002	4.19E-08
cg14938677	NO <sub>2</sub>	0.023	0.004	1.05E-08	1	0.023	0.004	1.05E-08
cg00344801	NO <sub>2</sub>	-0.028	0.005	2.38E-08	0			
cg18379295	NO <sub>2</sub>	0.020	0.004	3.50E-08	0			
cg25769469	NO <sub>2</sub>	0.035	0.006	3.69E-08	0			
cg02234653	NO <sub>2</sub>	-0.017	0.003	4.07E-08	12	-0.016	0.003	3.50E-07
cg08500171	NO <sub>2</sub>	0.023	0.004	9.81E-08	1	0.024	0.004	8.18E-08

Effect estimate per 10 µg/m<sup>3</sup> increase in NO<sub>2</sub> concentration

Robust linear regression analysis adjusted for sex, age, BMI, current smoking, pack-years, technical variance and blood cell composition

**Table S3.** Sensitivity analysis: Robust linear regression analysis for the association between NO<sub>2</sub> and DNA methylation at the 7 genome-wide significant CpG sites with additional adjustment for highest educational level, COPD, asthma, and respiratory medication use.

CpG	Original model n=1,017		Adjusted for highest educational level n=1,011		Adjusted for COPD n=1,017		Adjusted for Asthma n=1,014		Adjusted for use of respiratory medication n=1,017	
	B	p-value	B	p-value	B	p-value	B	p-value	B	p-value
cg04908668	-0.012	7.94E-09	-0.012	1.40E-09	-0.012	9.92E-09	-0.012	8.86E-09	-0.012	6.23E-09
cg14938677	0.023	1.05E-08	0.024	3.32E-09	0.023	1.51E-08	0.023	1.26E-08	0.023	1.94E-08
cg00344801	-0.028	2.38E-08	-0.028	7.29E-08	-0.028	3.48E-08	-0.028	2.28E-08	-0.029	1.96E-08
cg18379295	0.020	3.50E-08	0.021	1.70E-08	0.020	5.17E-08	0.020	2.57E-08	0.020	4.50E-08
cg25769469	0.035	3.69E-08	0.036	3.89E-08	0.035	3.87E-08	0.035	4.57E-08	0.034	6.09E-08
cg02234653	-0.017	4.07E-08	-0.017	3.98E-08	-0.017	5.39E-08	-0.017	6.24E-08	-0.017	6.30E-08
cg08500171	0.023	9.81E-08	0.024	6.16E-08	0.023	1.44E-07	0.024	1.14E-07	0.024	1.13E-07

Effect estimate per 10 µg/m<sup>3</sup> increase in NO<sub>2</sub> concentration

Robust linear regression analysis adjusted for sex, age, BMI, current smoking, pack-years, technical variance and blood cell composition

**Table S4.** Sensitivity analysis: Robust linear regression analysis for the association between NO<sub>2</sub> and DNA methylation at the 7 genome-wide significant CpG sites stratified by sex, BMI and smoking.

CpG	All (n =1,017)		Male (n= 587)		Female (n= 430)		Normal weight (n= 442)		Overweight (n= 575)		Never smoker (n= 574)		Current smoker (n= 443)	
	B	p-value	B	p-value	B	p-value	B	p-value	B	p-value	B	p-value	B	p-value
cg04908668	-0.012	7.94E-09	-0.013	7.39E-06	-0.011	6.12E-04	-0.013	1.61E-05	-0.010	2.49E-04	-0.010	4.02E-04	-0.014	1.02E-06
cg14938677	0.023	1.05E-08	0.026	5.90E-06	0.014	1.04E-02	0.021	4.06E-04	0.027	1.74E-06	0.024	4.15E-05	0.023	1.52E-05
cg00344801	-0.028	2.38E-08	-0.038	3.17E-07	-0.018	8.71E-03	-0.028	1.25E-04	-0.028	1.02E-04	-0.037	1.77E-06	-0.020	2.67E-03
cg18379295	0.020	3.50E-08	0.022	2.64E-05	0.016	1.94E-03	0.018	2.95E-04	0.022	2.82E-05	0.019	3.66E-04	0.023	5.72E-06
cg25769469	0.035	3.69E-08	0.040	2.30E-05	0.026	2.32E-03	0.030	6.67E-04	0.044	1.82E-06	0.038	6.93E-05	0.033	7.16E-05
cg02234653	-0.017	4.07E-08	-0.022	1.90E-06	-0.010	1.57E-02	-0.013	3.24E-03	-0.021	8.31E-07	-0.021	3.06E-05	-0.015	1.49E-04
cg08500171	0.023	9.81E-08	0.028	7.53E-06	0.017	9.84E-03	0.020	1.98E-03	0.028	8.47E-06	0.026	4.48E-05	0.024	1.34E-04

Effect estimate per 10 µg/m<sup>3</sup> increase in NO<sub>2</sub> concentration

Normal weight= BMI<25 kg/m<sup>2</sup>, Overweight= BMI≥25 kg/m<sup>2</sup>

Robust linear regression analysis adjusted for sex, age, BMI, current smoking, pack-years, technical variance and blood cell composition



**Table S5.** Significant associations between methylation levels at 2 genome-wide significant CpG sites and gene-expression of 3 genes (<http://www.genenetwork.nl/biosqtlbrowser/>)

CpG	Chr	Probe Name	HGNC Name	B ± SE	Overall p-value
cg04908668	6	ENSG00000240065	PSMB9	RS: -0.202 ± 0.039	2.10x10 <sup>-12</sup>
				LLD: -0.091 ± 0.040	
				LLS: -0.158 ± 0.039	
				CODAM: -0.167 ± 0.073	
cg04908668	6	ENSG00000168394	TAP1	RS: -0.201 ± 0.039	2.54x10 <sup>-8</sup>
				LLD: -0.032 ± 0.040	
				LLS: -0.123 ± 0.039	
				CODAM: -0.132 ± 0.073	
cg00344801	22	ENSG00000075234	TTC38	RS: 0.066 ± 0.039	1.85x10 <sup>-5</sup>
				LLD: 0.098 ± 0.040	
				LLS: 0.151 ± 0.039	
				CODAM: -0.030 ± 0.074	

RS: Rotterdam Study, LLD: LifeLines Deep cohort, LLS: Leiden Longevity Study, CODAM: Cohort on Diabetes and Atherosclerosis Maastricht.

**Table S6.** Association between PM10 exposure and DNA methylation in the LifeLines cohort study (based on  $p\text{-value} < 1 \times 10^{-5}$ ) (n=1,017).

CpG-site	Chr	Bp position	B (per 10 $\mu\text{g}/\text{m}^3$ PM10)	SE	p-value	Gene	Location in gene	Relation to Island
cg23876203	7	27159889	-0.057	0.012	1.65E-06	HOXA3	5'UTR;TSS1500	N_Shelf
cg07033961	5	760251	-0.062	0.013	1.82E-06			S_Shelf
cg14201224	9	133454820	0.012	0.003	2.33E-06	FUBP3	TSS200	Island
cg00668337	11	64491331	0.151	0.034	9.87E-06	NRXN2	TSS1500	S_Shore

Robust linear regression models adjusted for sex, age, body mass index (BMI), current smoking, pack-years, technical covariates, and blood cell composition.

**Table S7.** Association between PM<sub>2.5</sub> absorbance exposure and DNA methylation in the LifeLines cohort study (based on p-value<1x10<sup>-5</sup>)(n=1,017).

CpG-site	Chr	Bp position	<b>B (per 1*10<sup>-5</sup>/m PM<sub>2.5</sub><sub>abs</sub>)</b>	SE	p-value	Gene	Location in gene	Relation to Island
cg06920946	10	415489	-0.016	0.004	6.90E-06	DIP2C	Body	S_Shore
cg00668337	11	64491331	0.077	0.017	9.30E-06	NRXN2	TSS1500	S_Shore
cg25608871	15	79298384	-0.016	0.004	9.72E-06	RASGRF1	TSS1500,Body	OpenSea

Robust linear regression models adjusted for sex, age, body mass index (BMI), current smoking, pack-years, technical covariates, and blood cell composition.

**Table S8.** Robust linear regression analysis for the association between air pollution and lung function (n=1,017).

Variable	NO <sub>2</sub>		PM <sub>10</sub>		PM <sub>2.5</sub>		PM <sub>2.5</sub> absorbance	
	B (95% CI) <sup>a</sup>	p-value	B (95% CI) <sup>b</sup>	p-value	B (95% CI) <sup>c</sup>	p-value	B (95% CI) <sup>d</sup>	p-value
FEV <sub>1</sub> (mL)*	2.4 (-94.8, 99.6)	0.962	-433.5 (-1024.1, 157.2)	0.15	-491.5 (-1072.7, 89.6)	0.097	-206 (-504.5, 92.4)	0.176
FVC (mL)*	-106.3 (-219.1, 6.6)	0.065	-895.9 (-1582.8, -209.0)	0.011	-176.4 (-856.0, 503.3)	0.611	-492.2 (-839.6, -144.9)	0.006
FEV <sub>1</sub> /FVC (%) <sup>#</sup>	1.5 (-0.1, 3.0)	0.06	1.6 (-7.9, 11.0)	0.743	-11.4 (-20.6, -2.2)	0.016	1.5 (-3.3, 6.2)	0.547
FEF <sub>25-75</sub> (mL/s) <sup>\$</sup>	128.5 (-69.8, 326.8)	0.204	-160.5 (-1371.4, 1050.3)	0.795	-1329.3 (-2518.0, -140.6)	0.028	-0.9 (-614.8, 613.0)	0.998

<sup>a, b</sup> Effect estimates and 95% confidence interval (95% CI) for NO<sub>2</sub> and PM<sub>10</sub> exposures are given per 10 µg/m<sup>3</sup> increase

<sup>c</sup> Effect estimates and 95% confidence interval (95% CI) for PM<sub>2.5</sub> exposure are given per 5 µg/m<sup>3</sup> increase

<sup>d</sup> Effect estimates and 95% confidence interval (95% CI) for PM<sub>2.5</sub> absorbance exposure are given per 1x10<sup>-5</sup>.m<sup>-1</sup> increase

\* For FEV<sub>1</sub> and FVC, robust regression analysis adjusted for sex, age, height, BMI, sex\*age interaction, sex\*height interaction, current-smoking and pack-years

<sup>#</sup> For FEV<sub>1</sub>/FVC, robust regression analysis adjusted for sex, age, BMI, sex\*age interaction, current-smoking and pack-years

<sup>\$</sup> For FEF<sub>25-75</sub>, robust regression analysis adjusted for sex, age, height, BMI, sex\*age interaction, sex\*height interaction, current-smoking, pack-years and FVC

**Table S9.** Mediation analysis of DNA methylation in the association between NO<sub>2</sub> exposure and lung function.

CpG	AME <sup>a</sup>	95% CI	P-value	ADE <sup>b</sup>	95% CI	P-value	Total <sup>c</sup>	95% CI	P-value
Models of NO <sub>2</sub> exposure and FEV <sub>1</sub>									
cg04908668	-8.15	-24.80, 8.42	0.32	8.58	-83.79, 106.77	0.92	0.42	-89.61, 95.57	0.97
cg14938677	-3.22	-20.14, 11.91	0.75	4.20	-88.15, 100.32	0.97	0.98	-89.25, 94.30	0.98
cg00344801	1.62	-16.64, 20.59	0.83	0.17	-93.75, 92.96	0.98	1.79	-89.80, 95.27	0.99
cg18379295	1.94	-17.29, 20.34	0.83	-0.34	-91.77, 95.52	0.94	1.60	-88.97, 95.25	0.98
cg25769469	-4.28	-22.55, 13.49	0.64	4.99	-85.81, 101.41	0.94	0.71	-90.16, 94.21	0.98
cg02234653	-3.45	-17.73, 9.45	0.61	5.51	-88.74, 104.36	0.96	2.06	-89.70, 96.67	0.99
cg08500171	-3.15	-20.91, 14.06	0.71	4.31	-87.85, 102.06	0.97	1.16	-90.25, 94.57	0.98
Models of NO <sub>2</sub> exposure and FVC									
cg04908668	-9.23	-31.61, 9.31	0.33	-105.02	-229.57, 9.81	0.08	-114.25	-240.35, -3.79	0.04
cg14938677	-26.85	-50.85, -6.38	0.01	-91.82	-217.38, 16.91	0.11	-118.67	-244.41, -6.22	0.02
cg00344801	-14.14	-36.07, 4.30	0.15	-103.40	-227.01, 6.26	0.06	-117.53	-245.17, -7.91	0.03
cg18379295	-13.47	-37.15, 5.02	0.14	-102.00	-228.53, 9.56	0.07	-115.47	-241.62, -3.94	0.03
cg25769469	-13.91	-36.65, 4.63	0.15	-101.63	-226.55, 9.75	0.08	-115.54	-241.30, -4.28	0.04
cg02234653	-8.04	-24.35, 5.30	0.22	-105.68	-231.67, 4.17	0.06	-113.71	-238.13, -3.44	0.05
cg08500171	-17.58	-38.57, 1.76	0.07	-98.02	-219.98, 10.93	0.08	-115.60	-243.76, -4.64	0.03
Models of NO <sub>2</sub> exposure and FEV <sub>1</sub> /FVC									
cg04908668	0.02	-0.26, 0.30	0.90	1.46	-0.14, 3.11	0.08	1.47	-0.13, 3.16	0.07
cg14938677	0.29	0.01, 0.58	0.04	1.22	-0.36, 2.93	0.15	1.51	-0.07, 3.17	0.06
cg00344801	0.20	-0.06, 0.50	0.15	1.33	-0.22, 2.98	0.12	1.53	-0.10, 3.21	0.06
cg18379295	0.31	0.03, 0.66	0.03	1.24	-0.36, 2.90	0.14	1.55	-0.06, 3.25	0.06
cg25769469	0.10	-0.17, 0.42	0.48	1.39	-0.25, 3.05	0.09	1.50	-0.10, 3.18	0.07

cg02234653	0.08	-0.10, 0.27	0.40	1.39	-0.22, 3.08	0.10	1.46	-0.13, 3.14	0.08
cg08500171	0.17	-0.07, 0.45	0.18	1.33	-0.28, 2.99	0.13	1.50	-0.10, 3.21	0.06

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<sup>a</sup> AME: average mediation effect by DNA methylation

<sup>b</sup> ADE: average direct effect of NO<sub>2</sub> exposure on lung function

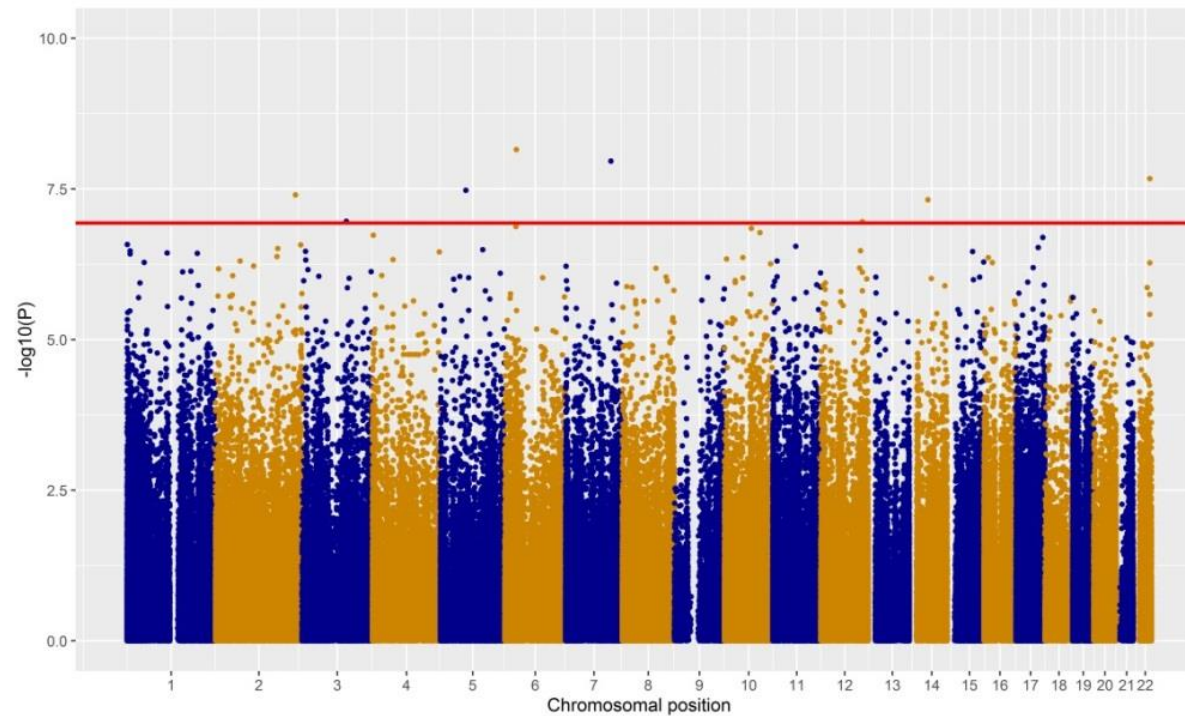
<sup>c</sup> Total: total effect of NO<sub>2</sub> exposure on lung function

Effect estimate per 10 µg/m<sup>3</sup> increase in NO<sub>2</sub> concentration

Robust linear regression models adjusted for sex, age, BMI, sex\*age interaction, current-smoking, pack-years, technical variance and blood cell composition, models for FEV<sub>1</sub> and FVC were additionally adjusted for height and sex\*height interaction

**Table S10.** Putative function of all genes annotated to the 7 genome-wide significant CpG sites (ordered by p-value) identified in the genome-wide DNA methylation study of NO<sub>2</sub> exposure.

CpG-site	Chr	Gene	Function (information obtained from <a href="http://www.genecards.org">www.genecards.org</a> )
cg04908668	6	PSMB9	<i>PSMB9</i> is involved in the major histocompatibility complex (MHC). It was found to be differentially expressed in lung cells of COPD-patients (Fujino et al. 2012).
cg14938677	7	ARF5	<i>ARF5</i> encodes proteins that activate phospholipase D (PLD), a critical enzyme involved in various endothelial and epithelial cell functions (Jenkins and Frohman 2005). Redox regulation of PLD plays a role in the interstitial pulmonary fibrosis (Patel et al. 2011).
cg00344801	22	TTC38	<i>TTC38</i> is a protein coding gene. It is broadly expressed in human tissues and was reported to be differentially expressed in monozygotic twin pairs discordant for current smoking (Vink et al. 2015).
cg18379295	14	GNG2	<i>GNG2</i> underlies important pathways such as cell migration, proliferation, differentiation, apoptosis and responses to external signals (Olate and Allende 1991). It is involved in airway hyper-responsiveness and inflammation in asthma model (Nino et al. 2012). <i>GNG2</i> is associated with regulation of the MAPK kinase cascade pathway (Yamauchi et al. 2001).
cg25769469	5	PTCD2	<i>PTCD2</i> encodes proteins involved in the mitochondrial RNA metabolism. In mice, it is suggested to be involved in the etiology of heart failure (Xu et al. 2008).
cg02234653	2	AP1S3	<i>AP1S3</i> is a heterotetramer that mediates membrane trafficking between the post-Golgi network and the endosome. This gene is reported to be associated with pustular psoriasis (Setta-Kaffetzi et al. 2014).
cg08500171	6	BAT2	<i>BAT2</i> lies within the class III region of the MHC and is a candidate gene for the age-at-onset of insulin-dependent diabetes mellitus.



**Figure S1.** Manhattan-plot showing the association between NO<sub>2</sub> exposure and differential DNA methylation in whole blood of subjects from the LifeLines cohort study. Seven CpG sites were genome-wide significantly associated with NO<sub>2</sub> exposure based on the Bonferroni corrected threshold  $p\text{-value} < 1.19 \times 10^{-7}$  (horizontal line). Each dot represents a single CpG site based on the chromosome location.



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