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Acute changes in DNA methylation in relation to 24 h personal air pollution exposure measurements: A panel study in four European countries

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ABSTRACT

Background: One of the potential mechanisms linking air pollution to health effects is through changes in DNA-methylation, which so far has mainly been analyzed globally or at candidate sites.
Objective: We investigated the association of personal and ambient air pollution exposure measures with genome-wide DNA-methylation changes.
Methods: We collected repeated 24-hour personal and ambient exposure measurements of particulate matter ($PM_{2.5}$), $PM_{2.5}$ absorbance, and ultrafine particles (UFP) and peripheral blood samples from a panel of 157 healthy non-smoking adults living in four European countries. We applied univariate mixed-effects models to investigate the association between air pollution and genome-wide DNA-methylation perturbations at single CpG (cytosine-guanine dinucleotide) sites and in Differentially Methylated Regions (DMRs). Subsequently, we explored the association of air pollution-induced methylation alterations with gene expression and serum immune marker levels measured in the same subjects.
Results: Personal exposure to $PM_{2.5}$ was associated with methylation changes at 13 CpG sites and 69 DMRs. Two of the 13 identified CpG sites (mapped to genes KNDC1 and FAM50B) were located within these DMRs. In addition, 42 DMRs were associated with personal $PM_{2.5}$ absorbance exposure, 16 DMRs with personal exposure to UFP, 4 DMRs with ambient exposure to $PM_{2.5}$, 16 DMRs with ambient $PM_{2.5}$ absorbance exposure, and 15 DMRs with ambient UFP exposure. Correlation between methylation levels at identified CpG sites and gene expression and immune markers was generally moderate.
Conclusion: This study provides evidence for an association between 24-hour exposure to air pollution and DNA-methylation at single sites and regional clusters of CpGs. Analysis of differentially methylated regions provides a promising avenue to further explore the subtle impact of environmental exposures on DNA-methylation.

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Table 1
Characteristics of study participants.

Characteristic	Italy[a]	Netherlands[a]	Switzerland[a]	United Kingdom[a]	Pooled[c]
Session (N samples)[b]					
1	43	41	48	23	155
2	42	40	44	20	146
Sex (N individuals)					
Female	22	34	25	15	96
Male	21	7	23	8	59
Education (N individuals)					
Secondary school	30	7	4	11	25
University	13	34	44	12	103
Age (years; median and P_{25}–P_{75})	60 (56–63)	62 (56–68)	60 (53–68)	63 (57–65)	61 (55–66)
BMI (kg/m^2; median and P_{25}–P_{75})	24.8 (22.5–26.6)	24.6 (22.6–27.1)	25.1 (21.6–26.9)	26.8 (24.3–29.3)	25 (22.5–27.9)
Physical activity (MET; median and P_{25}–P_{75})[d]	1.64 (1.5–1.7)	1.65 (1.5–1.7)	1.46 (1.4–1.6)	1.62 (1.5–1.8)	1.6 (1.5–1.7)
Season (N samples)					
1: spring (21/3–20/6)	34	18	35	15	102
2: summer (21/6–20/9)	16	31	18	16	81
3: autumn (21/9–20/12)	0	32	7	12	51
4: winter (21/12–20/3)	35	0	32	0	67

[a] In Italy individuals lived in Turin, in the Netherlands individuals lived in Utrecht and Amsterdam, in Switzerland individuals lived in Basel, and in the United Kingdom individuals lived in Norwich.
[b] Personal exposure monitoring and blood collection were performed twice for each individual. Session 1 and 2 were separated by a couple of months (in average 28 days (P_{25}–P_{75}): (20–37) between repeated sample collection).
[c] Our study population consisted of 157 non-smoking, healthy adults. Of which 144 individuals had two measurements and 13 individuals had one measurement. In total, 301 samples were analyzed and included in the statistical analyses.
[d] Physical activity was measured using the accelerometer (Actigraph GT3X+) and expressed in the 'The Metabolic Equivalent of tasks' (MET).

1. Introduction

Air pollution is now the world's largest single environmental health risk, leading to approximately 9 million deaths in 2015 alone (16% of all deaths worldwide) (Landrigan et al., 2017). The death burden relates mostly to the risk of cardiovascular and respiratory diseases (Brunekreef and Holgate, 2002; Hoek et al., 2013) and to lung cancer (Loomis et al., 2013). Oxidative damage, inflammation, and endothelial dysfunction have been suggested as potential underlying mechanisms (Demetriou et al., 2012; Pettit et al., 2012), though the identity and the role of potentially involved biological pathways are far from clear. It has been proposed that systemic effects of air pollution might be detected through assessing variation in epigenetics which can provide further mechanistic insights in air-pollution-health associations (Baccarelli et al., 2009; Herceg et al., 2017; Nawrot and Adcock, 2009). The most studied and best understood epigenetic modification is DNA-methylation, the covalent addition of a methyl group to a cytosine primarily in the context of a cytosine-guanine dinucleotide (CpG) (Bellavia et al., 2013; De Prins et al., 2013).

Early studies have provided indications for an impact of air pollution on peripheral blood global methylation using repetitive elements in long (LINE-1) and short (ALU) interspersed nuclear element (Baccarelli et al., 2009; Bellavia et al., 2013; De Prins et al., 2013). Furthermore, sequence-specific analyses have yielded evidence for differential methylation in regions of genes such as inducible nitric oxide synthase (iNOS), tissue factor intercellular adhesion molecule 1, toll-like receptor 2, interferon-y, and interleukin-6 (Bind et al., 2014; Madrigano et al., 2012; Tarantini et al., 2009; Wang et al., 2016).

In recent years, several cohort studies have conducted peripheral blood methylome-wide association studies of air pollution using the Illumina 450 BeadChip platform (Gruzieva et al., 2016; Panni et al., 2016; Plusquin et al., 2017). The KORA F3, F4 cohort (N = 2300 individuals), the Normative Aging Cohort (N = 657 individuals), the Multi-Ethnic Study of Atherosclerosis (MESA) (N = 1207 individuals), and the EPIC study (N = 613 individuals) identified several sets of CpG sites at which levels were associated with exposure to air pollution (Chi et al., 2016; Panni et al., 2016; Plusquin et al., 2017). In addition, a meta-analysis of multiple European and North American studies identified a set of three CpGs for which methylation levels in children were related to air pollution exposure (NO_2) of the mother during pregnancy (Gruzieva et al., 2016). To estimate exposure to air pollution, these methylome-wide studies used either spatially resolved models that predict long-term (1 year) average levels (MESA, EPIC), or temporally resolved (2-day, 7-day, and 28-day trailing averages) estimates based on citywide monitoring data (KORA). The relation between 24-hour personal measurements of air pollution and peripheral blood methylome-wide changes is currently unknown.

Our study contributes to the existing literature by providing insight into the relation between 24-hour personal measurements of particulate matter (PM) smaller than 2.5 μm ($PM_{2.5}$), $PM_{2.5}$ absorbance (as a proxy of black carbon and soot), and ultrafine-particulates (UFP) and genome-wide changes in the peripheral blood methylome. For comparison purposes, our study also incorporates modeled long-term average estimates of the same pollutants.

Associations with air pollutants were identified through genome-wide univariate screening of the peripheral-blood methylome and by analyses of differentially methylated regions (DMRs). PM from different pyrogenic sources may have toxicological similarities, we, therefore, assessed the overlap between our results and a set of CpG sites previously reported to be associated with cigarette smoke. In addition, to gain a better understanding of the functional role of differential methylation at air pollutant related CpG sites, we investigated their association with expression data of closely located genes and a set of 13 immune markers measured in the same individuals.

2. Material and methods

2.1. Study population

Our study, which is part of the EXPOsOMICS project (Vineis et al., 2016), was conducted in four European countries (Italy, Netherlands, Switzerland, and United Kingdom) from December 2013 to February 2015. We aimed to recruit 40 individuals from each country of whom half lived on busy roads (road with > 10 K vehicles per day; house on ground/first floor) and the other half on quiet roads (at least 100 m away from busy roads) in order to increase contrast in air pollutant levels. 157 healthy never-smoking adults between 50 and 70 years old, with no history of a pulmonary or cardiovascular disease, diabetes, or

other acute or chronic health conditions participated in our study (Table 1). Participants performed three personal exposure monitoring (PEM) sessions in different seasons (summer, winter, and spring/autumn) spread over one year (on average 28 days (P_{25}–P_{75}): (20–37) between repeated sample collection). During each PEM session, air pollution measurements and blood samples were collected for each subject. Subjects performed their own daily routine and filled in a questionnaire on food intake and time-activity. Blood samples from the first two PEM sessions were analyzed. Ethics approval for each country was obtained from local authorized review boards. All subjects signed an informed consent before participation.

2.2. Exposure assessment

During each PEM session, participants carried a backpack containing air pollution sensors to measure 24-hour personal air pollution exposure and a belt to measure locations (GPS) and an accelerometer. Simultaneously, with the personal measurements, 24-hour air pollution was measured outdoor at the subject's home address.

2.2.1. $PM_{2.5}$

For the personal measurements, 24-hour $PM_{2.5}$ was sampled on a 2 μm pore size Teflon filter, using a BGI 400 pump unit and GK 2.05 SH (BGI Inc., Waltham MA, USA) cyclone. Changes in filter weight (pre and post-weighted) measured in a central lab were used to assess $PM_{2.5}$ concentration. Ambient $PM_{2.5}$ was collected via installing the same devices as used for personal sampling, directly outside of the subject's home in the garden or balcony. Filter reflectance was measured to assess $PM_{2.5}$ absorbance concentration (soot levels) in both personal and home outdoor monitoring. Details on analytical procedures have been published previously (Eeftens et al., 2012).

2.2.2. Ultrafine particles (UFP)

Ultrafine particles (UFP) were continuously monitored with a MiniDiSC (Testo AG, Lenzkirch, Germany). The MiniDiSC operated at a flow of 1000 ml/min measuring particles from 10 to 300 nm at 1-second intervals. We used the term UFP to refer to the median of particle number counts from the MiniDiSC over 24-hour PEM session. Additionally, in The Netherlands and Switzerland ambient UFP was collected over each 24-hour PEM session at the subject's home facade using MiniDiSCs. Details on methods and quality control have been published previously (van Nunen et al., 2017).

2.2.3. Average exposure and long-term modeled exposure

In addition to the main personal exposure metrics, reflecting exposure to air pollution in the 24 h before blood collection, we calculated the average of the 24-hour personal air pollution measurements across the three PEM sessions as a proxy for long-term average personal exposure. We also used estimated annual average ambient concentrations of air pollution for each individual using the land-use-regression (LUR) prediction models of the ESCAPE study (European study of cohorts for air pollution effects) and EXPOsOMICS (Eeftens et al., 2012; van Nunen et al., 2017) as a proxy for long-term modeled ambient exposure. Further details are provided in the Supplemental materials '1'.

2.2.4. Physical activity

During each PEM session, physical activity was continuously measured with an accelerometer (Actigraph GT3X+, Pensacola FL, USA), which was attached to the participant's belt. We estimated energy expenditure in metabolic equivalent of task (MET) values from the raw acceleration data to express participant's physical activity over 24 h before blood collection (Ainsworth et al., 2000).

2.3. Biological sample collection

Biological samples were collected in the morning (9:00–11:00) after each PEM session. Collected blood samples were processed, aliquoted, and transferred to −80 °C within 2 h.

2.3.1. DNA-methylation measurements

Genome-wide DNA-methylation analyses were performed on peripheral blood samples (buffy coats) using the Illumina Infinium HumanMethylation450 BeadChip (HM450K), with optimized robotic pipelines (Ghantous et al., 2014; Morris and Beck, 2015). The HM450K array measures DNA-methylation at 485,512 cytosine positions across the human genome (CpG). Samples were randomized within each country. All laboratory procedures were carried out at the Epigenetics Group, International Agency for Research on Cancer (IARC, Lyon, France) according to manufacturers' protocols. CpG sites with > 10% missing methylation values were replaced by the average methylation level of that particular CpG site. After quality control exclusions, 459,333 CpGs were selected for further analyses. The percentage of methylation of a given cytosine (β-value) corresponds to the ratio of the methylated signal over the sum of the methylated and unmethylated signals. The M-value was calculated as the logit transformation of the β-value. The M-value was used in the statistical analysis because it is less heteroscedastic and performs better in differential methylation analysis (Du et al., 2010). For each CpG, extreme values were considered as outliers if they fell out of the interquartile range (IQR) (2.5th percentile $-3 \times$ IQR, 97.5th percentile $+3 \times$ IQR) interval and were removed from further analysis. The maximum percentage of outlier samples was 3.7% in < 10% of methylation markers.

DNA-methylation sites were mapped to genes based on data provided by Illumina (Bibikova et al., 2011) (for the individual probes) and on the February 2009 human reference sequence database (GRCh37) using the UCSC genome browser (Meyer et al., 2013; Pedersen et al., 2013) (both for the individual probes and the DMRs). Functional analyses of mapped genes that were identified in either the univariate analysis or DMRs to be associated with air pollution were assessed using NIH-DAVID bioinformatics resources (Huang et al., 2009).

2.3.2. Gene expression

Total RNA was isolated from stabilized blood specimens (400 μl of whole blood and 1600 μl of RNA later) using RiboPureTM-Blood (Ambion), according to the manufacturer's instructions. RNA from 301 samples was hybridized on Agilent 8 × 60 K Whole Human Genome microarrays for mRNA. Raw data on pixel intensities were extracted using Agilent Feature Extraction Software. After quality control exclusion, a total of 23,557 probes were available for the subset (245 of 301) samples. Further details are provided in the Supplemental materials '2'.

2.3.3. Immune markers assessment

A panel of 23 immune markers was measured in serum samples of all subjects using an R&D Systems (Abingdon, UK) Luminex screening assay. The panel included interleukins (IL) 1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-25, tumor necrosis factor alpha (TNF-α), eotaxin, IL1 receptor antagonist (IL1ra), CXC chemokine ligand 10 (CXCL10), epidermal growth factor (EGF), fibroblast growth factor beta (FGF-β), granulocyte colony-stimulating factor (G-CSF), melanoma growth stimulatory activity/growth-related oncogene (GRO), chemokine (C—C motif) ligand 2 (CCL2), C—C motif chemokine 22 (CCL22), macrophage inflammatory protein-1 beta (MIP-1β), vascular endothelial growth factor (VEGF), Myeloperoxidase (MPO), and periostin. In addition, C-reactive protein (CRP) was assessed using the R&D System Solid Phase ELISA. The panel of immune markers was a priori selected based on an informal review of the literature on air pollution, asthma, CVD, colon cancer, and lung cancer. This panel was applied in several studies part of the EXPOSOMICS project (Vineis et al., 2016).

After quality control assessment, 13 immune markers remained for further analysis. Immune markers were log-transformed as the distributions were skewed. Further details are provided in the Supplemental material '3.1'.

2.4. Data analysis

2.4.1. Univariate mixed-effects model

We used linear mixed effect models setting a random intercept (i) for each subject, capturing the correlation among measurements within the same subject (N = 2 observation per subject), (ii) for each microtiter plate (N = 37) and (iii) for the position of the sample on the chip (N = 12), capturing technically-induced variation potentially biasing DNA-methylation measurements. To control for potential confounding, models were adjusted for sex, age, body mass index (BMI) (kg/m^2), education (primary, secondary school, and university), country (Italy, Netherlands, Switzerland, and United Kingdom), season and physical activity expressed in MET-values. In addition, temperature and relative humidity as proxies of meteorological conditions were included in all models.

A number of sensitivity analyses were conducted. First, we ran a minimally adjusted model (only age, sex, and BMI included as covariates) and a model in which country was excluded from the primary set of covariates. To explore the possible influence of country, we ran our original model for each country separately and on the pooled set of data leaving data from one country out one at the time. We explored the stability of the methylation levels over time by using the random effects part of the mixed model to estimate intra-class correlation coefficients (ICC; the proportion of the total variance in the CpG sites that was due to variation between subjects, and not due to variation between plates, position on the plates, or error).

We also assessed the association between DNA-methylation and long-term average personal exposures as well as long-term modeled exposure to air pollution in two separate models with the same set of covariates as were considered in the original model. Missing values in covariate and exposure data were imputed using multivariate imputation by chained equations (MICE) in R (Buuren and Groothuis-Oudshoorn, 2011). No covariate had > 4% missing data (except for MET; 13%). In exposure data, 6.3% of personal $PM_{2.5}$ and 5.6% of ambient $PM_{2.5}$ values were missing. Since the proportion of missing data was low, only one imputation iteration was used in association analysis.

White blood cell composition was estimated based on the DNA-methylation data, using the Houseman algorithm (Houseman et al., 2012). Potential confounding by white blood cell composition was evaluated by assessing the association between white blood cell composition and the air pollutants using the linear mixed-models as described above.

Multiple testing adjustments on the resulting p-values were performed using the false discovery rate method of Benjamini-Hochberg (BH-FDR) (Benjamini and Hochberg, 1995) at the level of 0.05. Univariate mixed-effects models and imputation were conducted using R version 3.0.2 (packages: lme4, mice (De Boeck et al., 2011; Buuren and Groothuis-Oudshoorn, 2011)).

2.4.2. Differentially methylated regions (DMRs)

We identified DMRs using the comb-p package in Python (version 2.7.13) (Pedersen et al., 2012). p-Values for each probe generated by the univariate linear mixed-effect models (ordered by chromosomal location) were used as input. We set a window size of 300 bases and restricted DMRs to those that included at least 2 probes. A Stouffer–Liptak–Kechris (SLK) and Šidák corrected p-value < 0.05 was used as the statistical cut-off for significance. This resulted in regions of adjacent probes with low p-values that stand up to genome-wide correction. Regions were annotated to nearest gene and CpG island from human genome version "hg19" using the CruzDB software package (Meyer et al., 2013; Pedersen et al., 2013). A further description of this analysis is provided in Supplemental material '4'.

2.4.3. Overlap with CpG sites associated with cigarette smoking

We assessed the distribution of CpG sites associated with personal $PM_{2.5}$ exposure among a reference set of CpG sites associated with "exposure to cigarette smoke" (Joehanes et al., 2016) using the gene set enrichment methodology (Mostafavi et al., 2017; Subramanian et al., 2005). A p-value < 0.05 was used as the statistical cut-off for enrichment. A further description of these reference sets is provided in the Supplemental materials '5.1'.

2.4.4. Impact of air pollution on immune markers

We assessed the association between air pollution measurements and immune markers (n = 13) using univariate mixed-effects models. We included random intercepts for each subject which captures the correlation among measurements within the same subject and a random intercept for each microtiter plate (N = 9) which captures the nuisance variation generated in the assessment of the immune markers in the model. The same set of covariates as we defined in our primary univariate methylation models were used to control for potential confounding variables. Moreover, the same sensitivity analysis as we described in the methylation analysis was performed to assess the stability of our findings to variation in the confounder models. A further description of these analyses is provided in the Supplemental materials '3.2'.

2.4.5. Functional readout of the impact of differential methylation at the identified CpG sites

We assessed interrelationships between methylation levels at significant CpG sites identified in the methylation analysis (both univariate and DMRs analysis for each air pollutants separately; 415 CpG sites), expression of mapped genes (n = 58 transcripts), and the immune markers (n = 13) incorporated in this study using the XMWAS package (Uppal et al., 2017). We set the minimum pairwise correlation threshold to 0.4 and used a canonical Partial Least Squares regression (10 components) to generate pair-wise similarity matrices (González et al., 2012). We used a clustering approach to address the paired measurements in this analysis. Connections with a correlation < 0.4 were not shown in the figure. The correlation structure between the three platforms was displayed using the Cytoscape software environment (Shannon et al., 2003).

3. Results

Baseline characteristics of the study participants are presented in Table 1. Our study population consisted of 157 non-smoking, healthy adults for whom 301 blood samples and air pollutant measurements were collected across two sampling campaigns (144 participants with two measurements and 13 participants with one measurement). About 61% of participants were female and > 65% of participants had a university or college degree. The population had a median (P_{25}–P_{75}) age of 61 (55–66) years.

In Fig. 1, we show the distribution of 24-h average personal and ambient air pollution measurements by country. Highest air pollutant levels were measured in Italy, followed by The Netherlands. Lower levels were measured in the United Kingdom and in Switzerland (Fig. 1).

We observed a relatively high correlation between measured ambient and personal air pollution concentration measurements, especially for $PM_{2.5}$ absorbance (r = 0.74). Correlations between short-term measured air pollution concentrations and modeled long-term air pollution concentrations were low. Correlations between UFP and the other air pollutants were weak to moderate (r = 0.18; Fig. S1).

We did not observe any significant association between the air pollutants and estimated white blood cell type composition (Table S2); therefore, adjustment for white blood cell type composition was not used in further analyses.

3.1. Univariate analyses

We identified 13 CpG sites (Mapped to 14 genes) at which

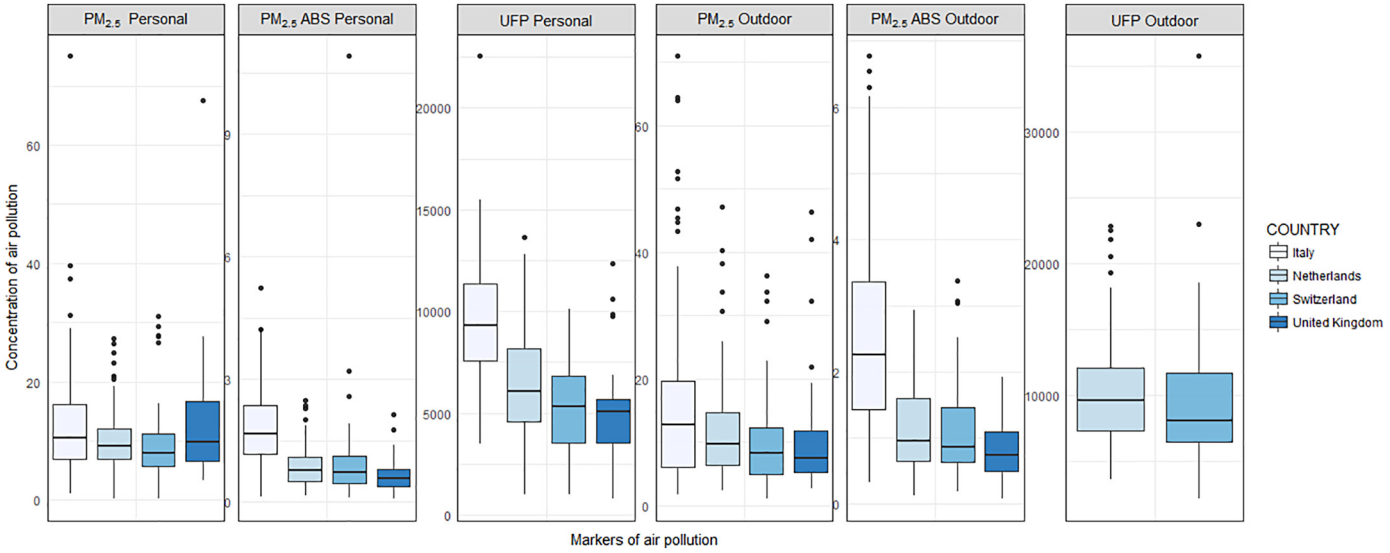

Fig. 1. Box plots of air pollution concentrations by country. Each panel shows one air pollution marker; personal $PM_{2.5}$; $\mu g/m^3$, personal $PM_{2.5}$ absorbance; m^{-1}, personal UFP; particles/cm^3, ambient $PM_{2.5}$; $\mu g/m^3$, ambient $PM_{2.5}$ absorbance; m^{-1}, and ambient UFP; particles/cm^3. Each box represents one country (Italy, Netherlands, Switzerland, and the United Kingdom). Horizontal lines correspond to medians, and boxes to the 25th–75th percentiles; whiskers extend to data within the interquartile range times 1.5, and data beyond this are plotted as dots.

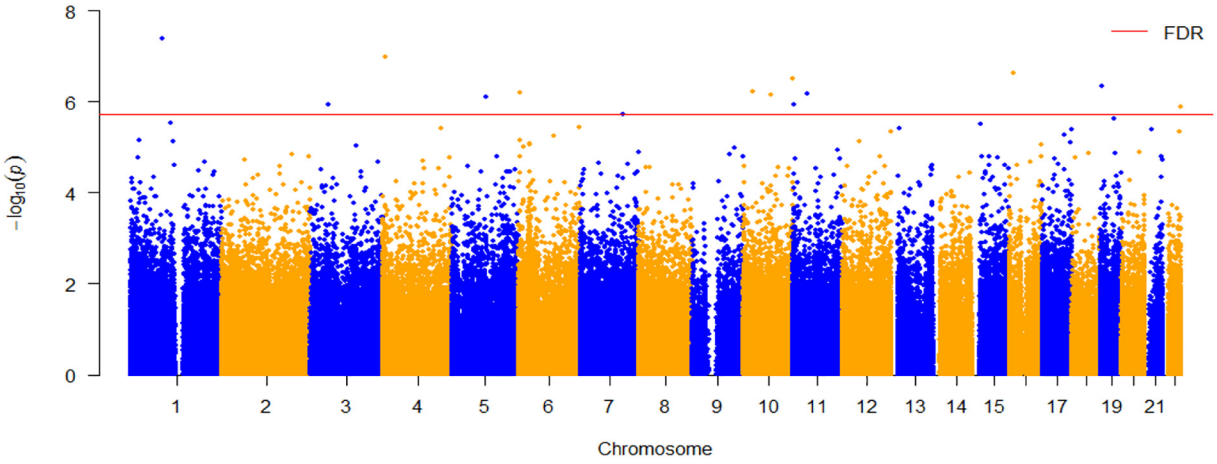

Fig. 2. Manhattan plot showing fixed-effect p-values of association between personal $PM_{2.5}$ measurements and DNA-methylation for fully adjusted model. Each dot corresponds to the CpG methylation site. Horizontal lines correspond to adjusted p-value (FDR) at 0.05 level. 13 CpG sites above the line were considered statistically significant.

Table 2
CpG sites significantly (at FDR 5%) associated with personal measurements of $PM_{2.5}$.

Probe ID (CpG)	β[a]	p-Value	FDR	Chromosome	Location	Mapped gene (region)[b]	Relation to CpG island	Nearby genes[c]
cg26692818	0.23	4.1E−08	0.02	1	87,108,066	CLCA3P (gene body)	OpenSea	CLCA3P; LOC105378828
cg02556634	0.06	1.0E−07	0.02	4	6,449,000	PPP2R2C (gene body)	OpenSea	PPP2R2C
cg03873392	−0.13	2.4E−07	0.03	16	10,801,987		OpenSea	TEKT5
cg07669973	−0.12	3.0E−07	0.03	10	135,029,365	KNDC1 (gene body)	Island	KNDC1
cg02508204	0.20	6.8E−07	0.04	11	39,367,436		OpenSea	LINC01493
cg19850855	0.20	7.8E−07	0.04	5	93,333,655	FAM172A (gene body)	OpenSea	FAM172A
cg20673255	0.14	4.7E−07	0.04	19	5,787,465	DUS3L (gene body)	N_Shore	DUS3L
cg23468453	0.23	7.1E−07	0.04	10	73,906,532	ASCC1 (gene body)	OpenSea	ASCC1
cg26559703	0.24	6.1E−07	0.04	10	26,853,625	APBB1IP (gene body)	N_Shelf	APBB1IP
cg01905633	0.06	6.3E−07	0.04	6	3,849,391	FAM50B (TSS1500)	Island	FAM50B
cg03408122	0.13	1.3E−06	0.04	22	50,903,314	SBF1 (gene body)	Island	SBF1
cg05404940	−0.11	1.2E−06	0.04	11	1,446,911	BRSK2 (gene body)	OpenSea	BRSK2
cg20693615	0.23	1.2E−06	0.04	3	50,234,688	GNAT1 (3′UTR)	S_Shelf	GNAT1

[a] The coefficient estimate from a model with M values as the outcome.
[b] Annotations provided by Illumina (Bibikova et al., 2011).
[c] Annotation to nearest gene from human genome version hg19 with CruzDB package (Meyer et al., 2013; Pedersen et al., 2013).

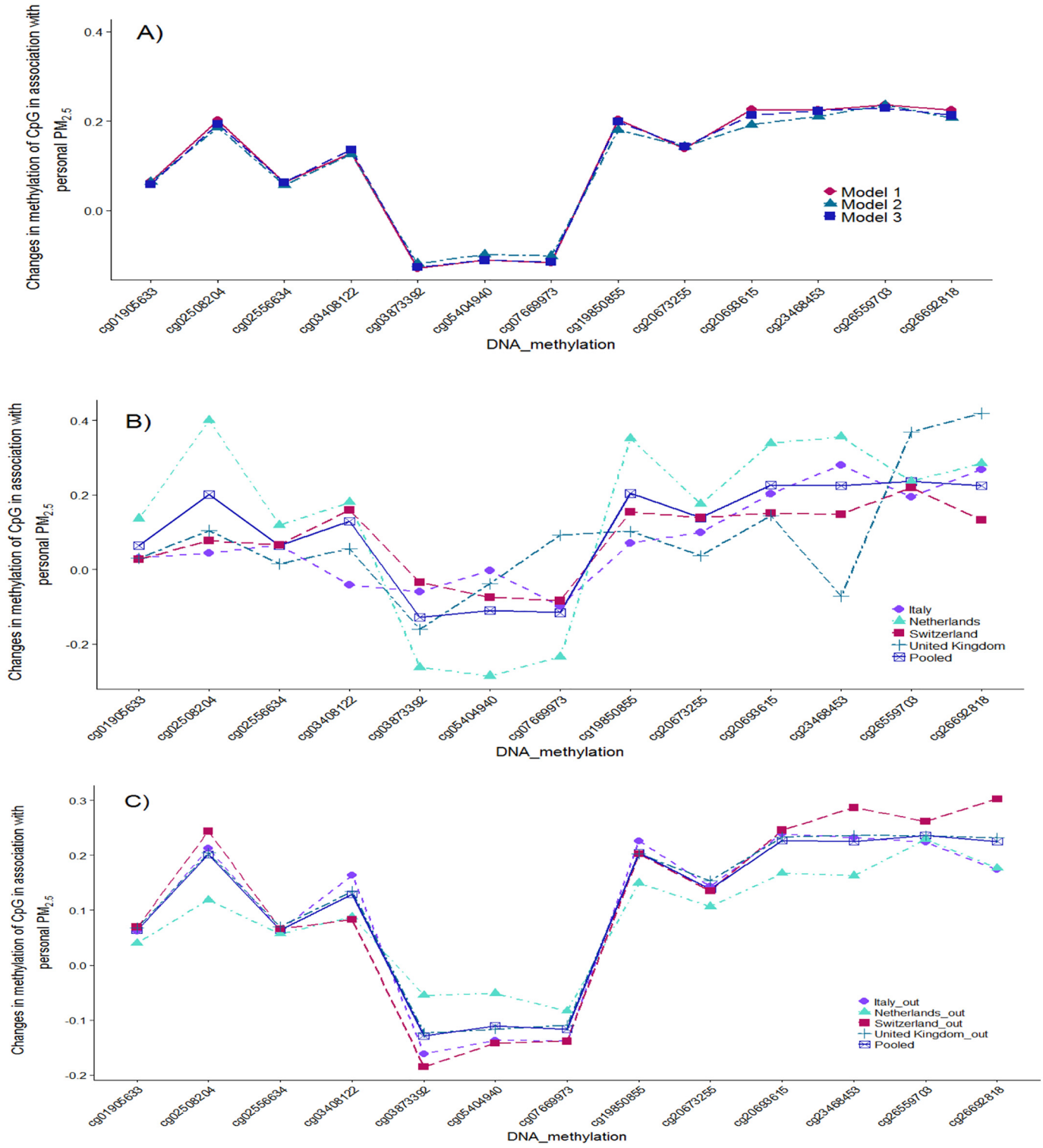

Fig. 3. (A) Association between personal $PM_{2.5}$ and methylation of 13 identified CpG sites for a different set of confounder adjustment including Model 1 (full model) adjusted for sex, age, BMI, education, season, physical activity, temperature, relative humidity, and country. Model 2 adjusted for sex, age, BMI, and country. Model 3 adjusted for all variables of Model 1 except for country. (B) Association between personal $PM_{2.5}$ and methylation of 13 identified CpG sites in pooled population and stratified by country. (C) Association between personal $PM_{2.5}$ and methylation of 13 identified CpG sites in pooled population and after leaving out countries one-by-one. In Italy individuals lived in Turin, in the Netherlands individuals lived in Utrecht and Amsterdam, in Switzerland lived in Basel, and in the United Kingdom lived in Norwich.

methylation levels were significantly associated (FDR < 0.05) with personal exposure to $PM_{2.5}$ (Fig. 2). For ten sites methylation increased with increasing exposure to $PM_{2.5}$, while for the remaining sites methylation decreased (Table 2). Between subject intraclass correlation for the 13 CpG sites was generally low (median 0.13), indicating considerable within individual variation. No clear correlation patterns in methylation levels were observed between the 13 CpG sites (Fig. S2). No significant associations were identified with any of the other

personal or ambient air pollution measurements (Table S3). However, coefficients for the 13 CpG sites associated with personal $PM_{2.5}$ in the models for the other air pollution markers were largely comparable reaching often nominal statistical significance at the $p < 0.05$ level, especially for personal $PM_{2.5}$ absorbance and ambient $PM_{2.5}$ (Table S4).

Sensitivity analyses indicated that the 13 associations found for $PM_{2.5}$ were robust to the set of confounders considered (Fig. 3A). Stratified analyses using data from each country separately yielded consistent effect size estimates in sign and magnitude (Fig. 3B). However, estimated strength of association was generally weakened upon stratification and revealed some heterogeneity across countries. Overall, of the 13 CpG sites identified in the pooled analysis, all were significantly associated to personal $PM_{2.5}$ in the Dutch population, 9 in the Basel population, 4 in the Turin population, and one in the Norwich population. The weakening of the strength of the associations can partially be attributed to the smaller sample size due to stratification. To account for this we ran a further sensitivity analysis excluding data from each country one-by-one. Results confirmed consistent effect size estimates with some evidence of stronger effects in The Netherlands for some of the CpGs (Fig. 3C).

Nominally significant ($p < 0.05$) effect estimates from a model that included the long-term average personal $PM_{2.5}$ exposure were correlated ($r = 0.77$; Fig. S3) to the nominally significant effect estimates from our main model based on 24-h personal measurements for $PM_{2.5}$, though none of the associations identified in this analysis were genome-wide significant. No correlation was observed with nominally significant effect estimates from a model that included long-term modeled exposure to $PM_{2.5}$ ($r = 0.02$; Fig. S3) and no genome-wide significant associations were identified.

3.2. Differentially methylated regions (DMRs)

We identified 162 DMRs (SLK-Sidak p-value < 0.05) of which 69 DMRs (containing 74 genes and 404 CpG sites) were significantly associated with personal exposure to $PM_{2.5}$ (Table 3), 42 DMRs (42 genes and 266 CpG sites) with personal exposure to $PM_{2.5}$ absorbance, 16 DMRs (21 genes and 109 CpG sites) with personal exposure to UFP, 4 DMRs (4 genes and 12 CpG sites) with ambient exposure to $PM_{2.5}$, 16 DMRs (19 genes and 142 CpG sites) with ambient $PM_{2.5}$ absorbance exposure, and 15 DMRs (20 genes and 125 CpG sites) with ambient exposure to UFP (Table S5 and Supplementary excel-file Tables E1, E2). Overlap of annotated genes across significant DMRs was observed between personal $PM_{2.5}$ and personal $PM_{2.5}$ absorbance ($n = 11$), and between personal $PM_{2.5}$ absorbance and ambient $PM_{2.5}$ absorbance ($n = 10$) while for other combinations overlap was less pronounced (Table S5). Two of the significant CpGs identified in our univariate analysis (Mapped to *KNDC1* and *FAM50B*) were located within the DMRs that were associated with $PM_{2.5}$.

3.3. Overlap with CpG sites associated with cigarette smoking

Cigarette smoke is a source of particulate exposure with some similar characteristics to ambient air pollution (but contributing to a much higher exposure). To assess the overlap between our results and a set of CpG sites previously reported to be associated to air pollution (Joehanes et al., 2016), we conducted an approach comparable to gene set enrichment (Subramanian et al., 2005). Hypermethylated CpG sites that were associated with cigarette smoking were significantly enriched (p-value < 0.01; Fig. S4A) among the most strongly hypermethylated CpG sites in our analysis for $PM_{2.5}$. A similar enrichment was observed for smoking-related hypomethylated CpG sites in our analysis ($p < 0.01$; Fig. S4B). Further description of these results is provided in the Supplemental materials '5.2'.

3.4. Impact of air pollution on immune markers

We identified a significant association between ambient exposure to $PM_{2.5}$ absorbance and serum concentration of CCL22 ($\beta = 0.11$; FDR = 0.03; Fig. S5A/B). The other pollutants yielded no significant association with any of the immune markers. Some indications of an association (FDR < 0.2) were observed between personal exposure measurements of UFP and serum concentration of two additional immune markers [CXCL10 ($\beta = 0.16$; FDR = 0.15) and G.CSF ($\beta = 0.32$; FDR = 0.15)], which are nevertheless noteworthy as comparable effects were observed for these markers in an independent population (PISCINA) that was part of the EXPOsOMICS project (Fig. S5A). The observed associations in that study were in the same direction as in our main study and significant (at FDR < 0.2) for G.CSF (0.25; FDR = 0.17) but not for CXCL10 (0.34; FDR = 0.28). The observed associations for CCL22, G.CSF, and CXCL10 were robust in a series of sensitivity analyses (Fig. S6). Further description of these results is provided in the Supplemental materials '3.3'.

3.5. Functional readout of the impact of differential methylation at the identified CpG sites

Fig. 4 shows the interrelations between CpG sites significantly associated to personal $PM_{2.5}$ (both in DMR (69 CpGs) and univariate analysis (13 CpGs)), expression of mapped genes, and the immune markers based on a similarity matrix generated using PLSR (González et al., 2012). Though modest in absolute correlation (absolute range of correlation was between 0.4 and 0.73), Fig. 4 illustrates a high degree of interrelatedness between the selected set of markers: 240 markers including 177 CpG sites (annotated to 56 unique genes), 55 expression of mapped genes (annotated to 41 unique genes), and 8 immune markers. We identified two clusters of considerably correlated markers and a very small weakly interrelated third cluster, which were primarily driven by the direction of the correlation. Cluster one and two contained 134 and 100 markers (nodes), respectively. Further information about clusters and interrelatedness is provided in Excel-file Table E4.

4. Discussion

Our study provides evidence that measurements of personal exposure to particulate air pollution, collected in a panel of healthy participants from four European countries, were associated with DNA-methylation changes in specific regions of the genome. Correlation between methylation at these CpG sites and a set of immune markers measured in peripheral blood was generally moderate, as was the correlation with the expression of genes mapped to the identified CpG sites. Several immune markers were independently associated with some of the air pollution measurements. Our results do not replicate findings from previous studies of short-term air pollution and blood DNA-methylation (Bellavia et al., 2013; Panni et al., 2016; Wang et al., 2018), though no consistent patterns have been observed in these studies. As we observed relatively low ICCs for the CpG sites that were associated to the $PM_{2.5}$ measurements, our study points towards (partly reversible) short-term variation of DNA methylation in peripheral blood in response to daily variation in $PM_{2.5}$ levels. Due to the ubiquitous presence of air pollution in our environment, studying the temporal behavior of the DNA methylome in response to exposure to air pollution in observational studies is a challenge.

A handful of experimental studies among both animals and humans do indicate a short-term response to exposure to air pollution. In one experimental study, demethylation of the interleukin-2 gene of lymph node T cells was observed in vivo 20 min after antigen presentation (Bruniquel and Schwartz, 2003). Furthermore, in a bioassay conducted by (Ding et al., 2017) methylation of LINE-1, iNOS, p16CDKN2A, and APC, in both lung tissue as well as in peripheral blood, changed after four hours of exposure to $PM_{2.5}$. (Bellavia et al., 2013) observed global

Table 3
DMRs associated with personal measurements of $PM_{2.5}$ at an SLK-Sidak p value of < 0.05.

DMR	Chromosome	Start	End	No. of probes	SLK-Sidak p value	Mapped gene[a]	Relation to CpG island
1	1	26,231,347	26,231,585	2	9.1E − 03	STMN1	Shore
2	1	167,408,509	167,409,199	7	5.6E − 03	CD247	Island
3	1	240,656,217	240,656,668	5	4.4E − 03	MIR1273E;GREM2	Island
4	1	153,599,479	153,600,157	8	1.8E − 02	S100A13	
5	1	92,414,221	92,414,911	8	3.1E − 03	BRDT	Island
6	1	244,952,269	244,952,395	3	3.2E − 02	COX20	
7	1	154,474,344	154,474,801	5	2.7E − 02	SHE;TDRD10	Island
8	1	153,234,037	153,234,388	4	1.3E − 02	LOR	
9	10	135,029,294	135,029,462	3	4.5E − 02	KNDC1	Island
10	10	14,051,636	14,052,029	6	9.6E − 03	FRMD4A	
11	10	70,321,554	70,321,960	6	2.1E − 03	TET1	Shore
12	11	1,413,145	1,413,316	3	2.2E − 02	BRSK2	Shore
13	11	1,456,891	1,457,347	4	7.0E − 03	BRSK2	
14	11	1,463,541	1,463,663	4	5.8E − 04	BRSK2	Shore
15	11	19,736,150	19,736,334	5	1.6E − 03	LOC100126784;NAV2	Shore
16	11	1,474,588	1,474,842	3	3.4E − 02	BRSK2	Shore
17	12	95,945,120	95,945,385	4	9.2E − 03	USP44	Shore
18	12	33,205,702	33,205,863	3	1.8E − 02	PKP2	
19	12	4,918,848	4,919,231	5	2.3E − 02	KCNA6	Island
20	12	71,552,188	71,552,570	4	7.3E − 03	TSPAN8	
21	12	46,319,990	46,320,111	4	3.1E − 02	SCAF11	
22	12	47,219,626	47,220,093	11	1.5E − 02	SLC38A4	
23	12	6,649,733	6,649,995	4	9.3E − 03	IFFO1	Island
24	15	83,240,550	83,240,792	7	2.5E − 02	CPEB1	
25	15	57,510,284	57,510,576	3	1.0E − 02	TCF12	
26	16	85,551,478	85,551,749	3	2.1E − 02	GSE1	Island
27	16	84,691,498	84,691,851	3	2.8E − 02	KLHL36	Island
28	16	89,408,076	89,408,568	6	3.0E − 02	ANKRD11	Island
29	16	67,686,832	67,687,120	3	2.0E − 03	CARMIL2	Island
30	17	33,759,484	33,760,250	10	7.9E − 06	SLFN12	
31	18	74,799,250	74,799,573	4	1.1E − 03	MBP	Island
32	19	45,448,959	45,449,302	5	4.7E − 02	APOC4-APOC2	
33	19	2,888,943	2,889,257	3	3.0E − 03	ZNF556	Island
34	19	55,660,514	55,660,626	5	2.3E − 02	TNNT1	
35	19	57,742,112	57,742,445	9	1.1E − 06	AURKC	Island
36	19	23,941,207	23,941,768	7	6.2E − 04	ZNF681	Island
37	19	11,649,462	11,649,702	5	2.2E − 02	CNN1	
38	19	45,975,694	45,976,196	5	4.2E − 02	FOSB	Island
39	19	38,281,047	38,281,560	5	9.4E − 04	ZNF573	Island
40	2	239,983,744	239,984,106	4	5.6E − 03	HDAC4	
41	2	207,506,480	207,507,164	9	3.0E − 02	FAM237A	Island
42	2	38,892,846	38,893,252	6	4.8E − 04	GALM	
43	2	20,870,812	20,871,402	6	6.2E − 07	GDF7	Island
44	2	64,868,515	64,868,711	2	1.9E − 03	SERTAD2	
45	2	242,763,794	242,763,983	2	8.7E − 03	NEU4	Shore
46	2	236,506,413	236,506,614	2	2.3E − 02	AGAP1	Island
47	2	20,870,087	20,870,363	3	1.1E − 04	GDF7	Island
48	20	26,190,328	26,190,355	3	1.9E − 02	MIR663AHG	Island
49	22	38,092,643	38,093,208	11	4.7E − 03	TRIOBP	
50	3	39,321,710	39,322,104	3	3.6E − 02	CX3CR1	
51	3	116,163,694	116,164,243	6	6.1E − 04	LSAMP	
52	4	74,847,646	74,848,017	8	1.2E − 04	PF4	Island
53	4	7,657,070	7,657,709	5	6.7E − 03	SORCS2	
54	4	174,429,263	174,429,542	6	4.0E − 02	SCRG1	Shore
55	4	46,995,203	46,995,744	7	1.9E − 03	GABRA4	Island
56	5	157,079,312	157,079,521	5	2.1E − 02	SOX30	Island
57	5	140,719,008	140,719,303	4	3.1E − 03	PCDHGA2;PCDHGA1	Island
58	5	1,962,311	1,962,555	3	9.1E − 04	CTD-2194D22.4	
59	6	31,938,984	31,939,547	12	5.5E − 04	DXO;STK19	Shore
60	6	3,849,190	3,849,703	21	7.0E − 04	FAM50B	Island
61	6	30,038,791	30,039,901	39	3.2E − 11	RNF39	Island
62	6	529,098	529,342	3	1.5E − 02	EXOC2	
63	6	126,080,132	126,080,724	4	3.9E − 04	HEY2	Island
64	7	158,905,317	158,905,537	3	1.9E − 02	VIPR2	Island
65	7	56,515,510	56,516,256	11	4.7E − 02	LOC650226	Island
66	8	599,963	600,556	6	2.8E − 06	ERICH1	Island
67	8	82,644,373	82,644,769	8	3.3E − 02	CHMP4C	Island
68	8	26,722,496	26,722,966	5	2.1E − 02	ADRA1A	Island
69	9	136,149,908	136,150,033	3	3.1E − 03	ABO	Island

[a] DMRs were annotated to nearest gene from human genome version hg19 with CruzDB package (Meyer et al., 2013; Pedersen et al., 2013).

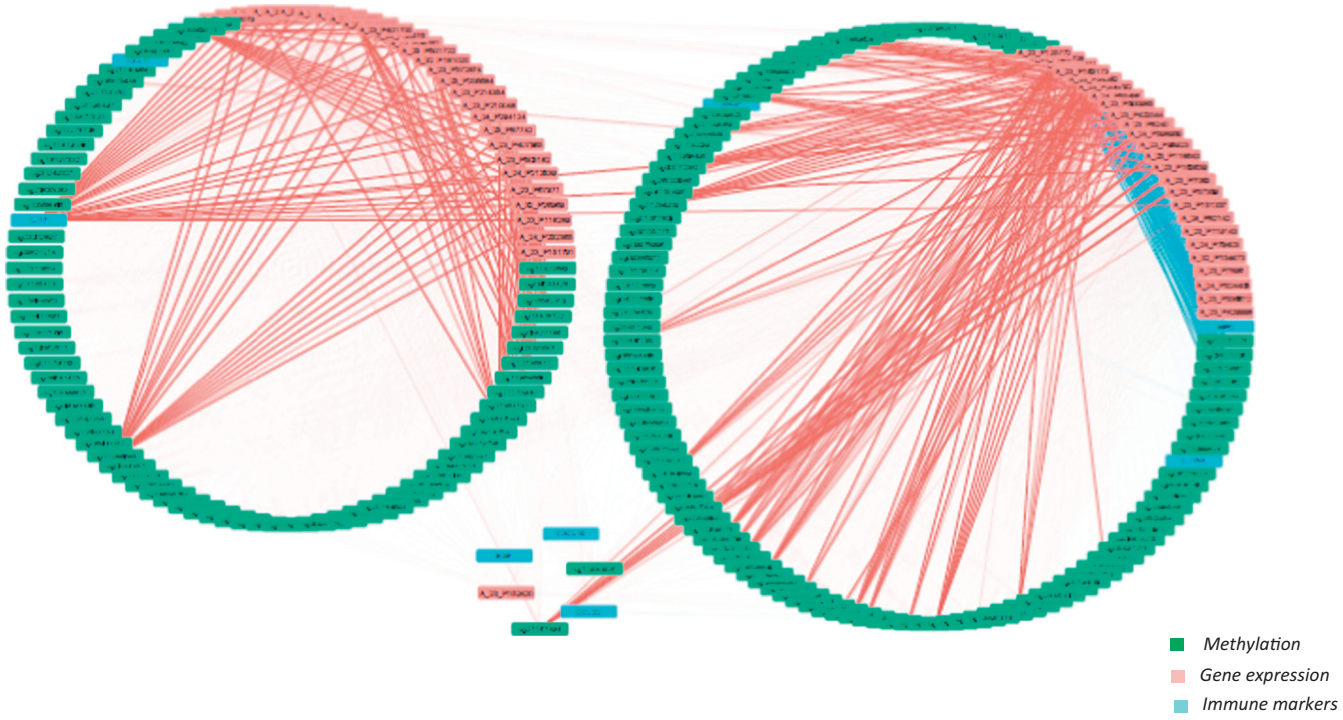

Fig. 4. Illustration of the interrelations between the three omics platform: Methylation (CpG sites significantly associated with personal $PM_{2.5}$ (both in DMR (69 CpGs) and univariate analysis (13 CpGs)); Green rectangle), immune markers (Blue rectangle) and gene expression (expression of mapped genes; Red rectangle). Three rings show different clusters (Cluster1 contained 134 omics markers and cluster 2 and 3, 100 and 6 omics markers, respectively. The connecting lines show association (correlation) between different markers (with absolutes range between 0.4, 0.73) Positive in Red; negative in Blue). The color intensity and the thickness of the lines both indicate the strength of the association (thicker lines indicate a correlation higher than 0.6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DNA demethylation after 130 min of exposure to fine concentrated ambient particles (Bellavia et al., 2013). (Chen et al., 2016) observed significant reduction of both global and site-specific methylation after 48 h of exposure to $PM_{2.5}$ (Chen et al., 2016). (Jiang et al., 2014) reported significant changes in DNA methylation 6 h after exposure to diesel exhaust (Jiang et al., 2014).

In addition to experimental evidence, several observational studies identified significant associations between short-term changes in methylation and exposure to air pollution. (Panni et al., 2016). observed demethylation to be associated with average air pollution levels in the two days before blood sampling (though a stronger signal was observed with longer trailing averages (Panni et al., 2016), while (Wang et al., 2018) observed demethylation within 12 h of exposure to $PM_{2.5}$ (Wang et al., 2018).

Combined, results from our and other studies suggest that the impact of air pollution on the blood methylome is likely subtle and requires high-quality exposure assessment and considerable statistical power to be studied.

Strengths of our study include the application of a common study protocol and standardized operating procedures applied across four European countries. DNA-methylation, gene expression, and immune markers were each analyzed in a single laboratory and blinded duplicate samples were included for quality assurance. State-of-the-art personal exposure measurements of air pollution were conducted to reduce the impact of measurement error and we repeated measurements within a time period of a year to cover seasonal variation. High-quality exposure assessment is resource intensive. Our study was, therefore, necessarily modest in the number of individuals that could be included in the study, which is a limitation. Another limitation of our study was the fact that we were unable to identify an independent dataset in which we could replicate the methylation findings of this study. However, we did assess the robustness of the identified signals within our dataset as much as possible.

In addition to univariate regression analysis, we applied two alternative approaches (gene set enrichment and DMR) that maximized the information we could derive from our study. Both approaches overcome statistical limitations in the assessment of perturbations by combining information from multiple CpG sites rather than assessing sites one by one. Enrichment of smoking-related CpG sites among the CpG sites most strongly associated with air pollution points towards a shared biologic pathway of the effects of cigarette smoke and air pollution on the epigenome. This is of interest due to overlap between health outcomes that have been related to tobacco smoking and air pollution (Wang et al., 2015). We recently discovered a similar overlap in an analysis of another air pollutant (NOx) and the peripheral blood transcriptome (Mostafavi et al., 2017). We focused on DMRs in our analysis because CpG sites mostly function in groups to regulate gene expression, rather than independently (Breton et al., 2017). Similar to gene set enrichment, our DMR analysis combined information from multiple CpG sites rather than assessing sites one by one and therefore also increased the statistical power to detect subtle perturbations in methylation patterns, leading to a much richer signal in our DMR analysis than in our univariate regression. Two CpG sites that were independently significantly associated to $PM_{2.5}$ were located within a DMR, which increased the credibility of these two associations.

Acknowledging that the 450 K array chip was designed to interrogate the methylation status of CpG sites in proximity to genes, a potential approach to gain insight into the biological role of DMRs and single CpG sites associated with air pollution involves mapping them to nearby genes and conducting functional enrichment analysis on these genes (Wright et al., 2016). Functional analysis of the identified CpG sites at the mapped genes using the NIH-DAVID bioinformatics resources (Huang et al., 2009) did not provide any insight into potentially affected biologic pathways, likely due to the limited number of CpG sites found in this study. Further functional interpretation of the identified CpG sites requires moving towards regulatory enrichment

analysis, incorporating not only mapping of nearby genes, but also acknowledging trans-acting effects of DNA-methylation on gene expression (Wright et al., 2016). In this study, we observe some evidence for such complex interactions between DNA-methylation, gene expression, and immune markers by showing the correlations between these markers. The existence of three correlation clusters could point towards shared biological function, though further analyses are required to confirm this interpretation.

24-hour personal exposure measurements of $PM_{2.5}$ were more strongly associated with DNA-methylation than long-term modeled exposure estimates of $PM_{2.5}$. Plusquin et al., using a much larger study among healthy adults, reported a similar absence of significant association of DNA-methylation in response to long-term $PM_{2.5}$, NO_x, NO_2 (Plusquin et al., 2017). These observations could be an indication that acute changes in methylation due to exposure to air pollution identified in this study did not reflect a long-term association between air pollution and DNA-methylation. However, long-term modeled exposure estimates could also be much more affected by measurement error which would have reduced the statistical power to detect significant associations.

24-h personal exposure measurements of $PM_{2.5}$ were also more strongly associated with DNA-methylation than ambient measurements of $PM_{2.5}$ and measurements of the other air pollutants. Measurements of ambient $PM_{2.5}$ and $PM_{2.5}$ absorbance were likely more strongly affected by measurement error. The weaker effect for personal $PM_{2.5}$ absorbance is surprising as $PM_{2.5}$ absorbance has been reported to be a very good marker for exposure to traffic-related particles (Cyrys et al., 2003). Direct comparison of the results for $PM_{2.5}$ with those for personal UFP measurements is complex as these pollutants not only differ in particle size but also in the way they were assessed (mass weight versus counts). Differences in observed associations might, therefore, be due to measurement error but might also have a biological interpretation related to particle size. Further work is required to clarify this issue.

The optimal way to summarize continuous UFP measurements in an aggregated metric is still an ongoing discussion (van Nunen et al., 2017). Exposure to UFP appears to be primarily determined by peaks and therefore summarizing exposure into a mean or median might not optimally reflect the impact of 24-hour exposure to UFP on the human system (van Nunen et al., 2017). The collected continuous UFP measurements provide opportunities for further explorations of sensitive time windows and the contribution of outdoor versus indoor sources to the total UFP exposure received (van Nunen et al., 2017).

In this study, we assessed exposure in the 24 h before blood draw. Limited information is available on the time-scale on which environmental factors are expected to have an impact on changes in DNA-methylation. Therefore the time-period over which exposure should be assessed to maximize the power to assess potential associations is not known. In a study by (Wang et al., 2016, 2018), personal monitoring of $PM_{2.5}$ was conducted 3 days before scheduled blood draw using a microPEM device. Based on this data different time lags were calculated, of which the 0–24 h time lag was most closely related to differential methylation. This result provides some support for the time window included in our study.

Even though we did not observe any associations between exposure to air pollution and estimated cell type composition, we repeated the main analysis for $PM_{2.5}$ correcting for the estimated cell type composition by including seven cell types ("Monocytes", "B", "CD4T", "NK", "CD8T", "Eosinophils", "Neutrophils"). Results did not change substantially (Table S6) and were strongly correlated ($r = 0.92$) with the effect estimates from our original model.

Our study also provides evidence for perturbation of serum concentration of three immune markers (CCL22, CXCL10, and G.CSF) in association with air pollution (CCL22 with ambient $PM_{2.5}$ absorbance; CXCL10 and G.CSF with personal UFP). So far, these immune markers have not been reported in association with air pollution markers in other studies. However, previously published reports on the association between short-term air pollutants and systemic inflammation in human study populations were inconsistent (Hassanvand et al., 2017; Larsson et al., 2013; Steenhof et al., 2014).

5. Conclusion

In conclusion, this study provide evidence for an association between 24-hour exposure to air pollution, in particular measurements of $PM_{2.5}$, and DNA-methylation both at single CpG sites and DMRs. Analysis of DMRs provides a promising avenue to further explore the subtle impact of environmental exposures on DNA-methylation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.07.026.

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