Occupational exposure to gases/fumes and mineral dust affect DNA methylation levels of genes regulating expression

Diana A. van der Plaat1,2, Judith M. Vonk1,2,4,†, Natalie Terzikhan3,4,#, Kim de Jong1,2,4,€, Maaike de Vries1,2,¶, Sacha La Bastide-van Gemert1,4,‡, Cleo C. van Diemen1,4,||, Lies Lahousse3,4,5,§, Guy G. Brussel6,3,4,5, Ivana Nedeljkovic4,§§, Najaf Amin4,¶¶, BIOS Consortium¶, Hans Kromhout7,##, Roel C. H. Vermeulen7, Roel C. S. Postma8,2, Cornelia M. van Duijn4 and H. Marike Boezen1,2,*

© The Author(s) 2019. Published by Oxford University Press.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

Human Molecular Genetics, 2019, Vol. 28, No. 15 2477–2485
doi: 10.1093/hmg/ddz067
Advance Access Publication Date: 2 April 2019
Association Studies Article

ASSOCIATION STUDIES ARTICLE

Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5. Data management and computational infrastructure: Hailiang Mei (Chair)12, Maarten van Iterson1, Michiel van Galen6, Jan Bot13, Dasha V. Zhernakova6, Rick Jansen7, Peter van’t Hof12, Patrick Deelen5, Irene Nooren13, Peter A. C. ‘t Hoen2, Bastiaan T. Heijmans (Chair)1, Peter-Bram ‘t Hoen (Co-Chair)2, Joyce van Meurs (Chair)3, P. MilaJhamai3, Michael Verbiest3, H. Eka D. Suchiman1, Marinj Verkerk1, Ruud van der Breggen1, Jeroen van Rooij3, Nico Lakenberg5.
Abstract

Many workers are daily exposed to occupational agents like gases/fumes, mineral dust or biological dust, which could induce adverse health effects. Epigenetic mechanisms, such as DNA methylation, have been suggested to play a role. We therefore aimed to identify differentially methylated regions (DMRs) upon occupational exposures in never-smokers and investigated if these DMRs associated with gene expression levels. To determine the effects of occupational exposures independent of smoking, 903 never-smokers of the LifeLines cohort study were included. We performed three genome-wide methylation analyses (Illumina 450 K), one per occupational exposure being gases/fumes, mineral dust and biological dust, using robust linear regression adjusted for appropriate confounders. DMRs were identified using comb-p in Python. Results were validated in the Rotterdam Study (233 never-smokers) and methylation-expression associations were assessed using Biobank-based Integrative Omics Study data (n = 2802). Of the total 21 significant DMRs, 14 DMRs were associated with gases/fumes and 7 with mineral dust. Three of these DMRs were associated with both exposures (RPLP1 and LINCO02169 (2×)) and 11 DMRs were located within transcript start sites of gene expression regulating genes. We replicated two DMRs with gases/fumes (VTRNA2-1 and GNAS) and one with mineral dust (CCDC14NL). In addition, nine gases/fumes DMRs and six mineral dust DMRs significantly associated with gene expression levels. Our data suggest that occupational exposures may induce differential methylation of gene expression regulating genes and thereby may induce adverse health effects. Given the millions of workers that are exposed daily to occupational exposures, further studies on this epigenetic mechanism and health outcomes are warranted.

Introduction

Daily, millions of workers worldwide are exposed to chemical agents, fumes and (in)organic dusts (1). The leading occupational causes of death in 2000 were unintentional injuries (41%), chronic obstructive pulmonary disease (COPD, 40%) and lung cancer (13%) (1). This is not remarkable, since the skin and the lungs are most directly exposed to occupational pollutants, which could be prevented by implementing protective measures. Studies focusing on specific occupations, like pig farmers, miners, construction and textile workers, found associations between job-specific exposures and a faster annual decline in lung function (FEV1) (2–4). In addition, we have previously shown that exposure to gases/fumes, mineral and biological dust is associated with small and large airways obstruction (5,6).

Even though occupational exposures are common, it is still largely unknown how these exposures are involved in (lung) disease development. Epigenetic mechanisms such as DNA methylation have been suggested to play a role, and researchers have therefore advocated the importance of epigenetic studies into environmental exposures and lung health (7). Environmental exposures, like occupational exposures, induce changes in DNA methylation levels, which can affect gene expression, possibly aiding in disease development (6). DNA methylation is the addition of a methyl group to the DNA without altering its sequence. Studies focusing on specific occupations, like pig farmers, miners, construction and textile workers, found associations between job-specific exposures and a faster annual decline in lung function (FEV1) (2–4). In addition, we have previously shown that exposure to gases/fumes, mineral and biological dust is associated with small and large airways obstruction (5,6).

Even though occupational exposures are common, it is still largely unknown how these exposures are involved in (lung) disease development. Epigenetic mechanisms such as DNA methylation have been suggested to play a role, and researchers have therefore advocated the importance of epigenetic studies into environmental exposures and lung health (7). Environmental exposures, like occupational exposures, induce changes in DNA methylation levels, which can affect gene expression, possibly aiding in disease development (6). DNA methylation is the addition of a methyl group to the DNA without altering its sequence. This usually occurs at sites where a cytosine base is adjacent to a guanine base (CpG) and can have a regulatory function on gene expression (9). Several small studies showed suggestive evidence that specific compounds found in occupational exposures, like cadmium, lead and mercury, affect DNA methylation (8,10–12).

To date, no large hypothesis-free genome-wide DNA methylation studies assessing the association between occupational exposures and DNA methylation levels have been performed. We therefore aimed to identify differentially methylated CpG sites (CpGs) and differentially methylated regions (DMRs) associated with occupational exposure to gases/fumes, mineral dust and biological dust, and to assess the effects of these regions on...
gene expression levels. To determine the effects of occupational exposures independent of smoking exposure, the analyses were restricted to never-smokers.

**Results**

**Population characteristics**

Our identification cohort comprised 903 never-smokers of the LifeLines cohort study with genome-wide DNA methylation data (Illumina 450 K) and complete data on all covariates (13). The validation cohort comprised 233 never-smokers of the Rotterdam Study and the characteristics of both cohorts are presented in Table 1, with additional characteristics of LifeLines subjects shown in Supplementary Material, Table S1 (14).

Three genome-wide methylation analyses were performed in never-smokers, one per occupational exposure being gases/fumes, mineral dust and biological dust, and consequently DMRs were identified. We present the results of our analyses per occupational exposure, and see Figure 1 for an overview of the performed analyses and corresponding results. The results of all analyses can be found in the supplementary Excel file including all supplementary tables and the Manhattan plots are shown in Supplementary Material, Figure S1.

**Gases/fumes**

**Genome-wide methylation analysis.** In the genome-wide methylation analysis in never-smokers of the identification cohort, two single CpGs were epigenome-wide significantly associated with gases/fumes exposure [false discovery rate (FDR) < 0.05] (Supplementary Material, Table S2). These CpGs are annotated to ribosomal protein L37a (RPL37A) and Grid2-interacting protein (GRID2IP).

**Identification of DMRs.** Thirteen DMRs were significantly associated with exposure to gases/fumes (Supplementary Material, Table S3). The three most significant DMRs are annotated to ribosomal protein L37a (RPL37A) and Grid2-interacting protein (GRID2IP).

**Validation of the DMRs.** In the validation analysis, two DMRs contained a significantly replicated CpG and exposure to gases/fumes was associated with lower methylation levels at these CpGs in both cohorts (Tables 2 and S4). These two DMRs are annotated to Vault RNA 2–1 (VTRNA2–1, a.k.a. MIR886) and guanine nucleotide-binding protein alpha stimulating activity (GNAS) (Fig. 2A and B).

**Gene expression analysis.** We found that CpGs within 9 out of 14 DMRs were significantly associated with differential gene expression, the direction of effect was predominantly negative. Table 3 presents the significant methylation-expression associations of CpGs within replicated DMRs. For the results of all DMRs, see Supplementary Material, Table S5. The replicated DMRs annotated to GNAS were associated with lower expression of NPEPL1.

**Mineral dust**

**Genome-wide methylation analysis and identification of DMRs.** No CpGs were genome-wide significantly associated with mineral dust exposure in our identification cohort (FDR < 0.05), but seven DMRs were (Supplementary Material, Table S3). The three most significant hits are annotated to RPLP1, LINC02169 and major histocompatibility Complex class I E (HLA-E), and the first two mentioned DMRs were also associated with exposure to gases/fumes.

**Validation of the DMRs.** The DMR annotated to coiled-coil domain containing 144 family, N-terminal like (CCDC144NL) contained a significantly replicated CpG and the association between mineral dust exposure and methylation levels was negative in both cohorts (Table 2, Supplementary Material, Table S4 and Fig. 2C).

**Gene expression analysis.** In total, CpGs within six out of seven DMRs were significantly associated with differential gene expression and the direction of effect was predominantly negative (Supplementary Material, Table S6). The replicated DMR annotated to CCDC144NL was associated with lower expression of abhydrolase domain containing 17A pseudogene 6 (ABHD17AP6), dehydrogenase/reductase 7B (DHRS7B) and galectin 9B (LGALS9B) (Table 3).

**Biological dust**

No single CpGs or DMRs were genome-wide significantly associated with biological dust exposure in never-smokers of the identification cohort (FDR < 0.05). Therefore, no validation of results or methylation-expression analyses was performed.

**Discussion**

This is the first genome-wide DNA methylation study assessing the association between occupational exposures and DNA methylation. Since it is well known that smoking is associated with extensive changes in DNA methylation levels, we restricted our analyses to never-smokers (15). In these never-smokers, occupational exposure to gases/fumes and to mineral dust was associated with 14 and 7 DMRs, respectively. Three of these DMRs were associated with both gases/fumes and mineral dust (one DMR in RPLP1 and two DMRs in LINC02169). We were able to replicate the result of two DMRs associated with gases/fumes, and one DMR was associated with mineral dust in the Rotterdam Study. These three DMRs were annotated to VTRNA2-1, GNAS.
| DMR         | Chr  | Start      | End        | Probes | N   | Annotation                  | Feature | Island | Region    | CpG     | Beta  | SE   | P         | Beta  | SE   | P         |
|-------------|------|------------|------------|--------|-----|----------------------------|---------|--------|-----------|---------|-------|------|-----------|-------|------|-----------|
| Gases/fumes | GN4  | chr5       | 135416331  | 135416579 | 7   | VTRNA2–1                   | TSS200  | Island | 2.68*10⁻⁵ | cg18678645 | -2.84 | 0.83 | 5.96*10⁻⁴ | -5.68  | 3.49 | 0.103     |
|             |      |            |            |         |     |                            |         |        |           | cg06536614  | -1.31 | 0.56 | 1.90*10⁻² | -3.25  | 2.47 | 0.188     |
|             |      |            |            |         |     |                            |         |        |           | cg26328633  | -1.88 | 0.59 | 1.50*10⁻³ | -5.81  | 2.67 | 0.029     |
|             |      |            |            |         |     |                            |         |        |           | cg25340688  | -1.96 | 0.63 | 1.80*10⁻³ | -2.04  | 2.89 | 0.479     |
|             |      |            |            |         |     |                            |         |        |           | cg26896946  | -1.10 | 0.44 | 1.18*10⁻³ | -2.23  | 2.33 | 0.318     |
|             |      |            |            |         |     |                            |         |        |           | cg00124993  | -1.59 | 0.55 | 3.75*10⁻³ | -6.23  | 3.32 | 0.061     |
|             |      |            |            |         |     |                            |         |        |           | cg08745965  | -2.21 | 0.84 | 8.84*10⁻³ | -6.87  | 4.50 | 0.127     |
|             | GN13 | chr20      | 57427713   | 57427880 | 6   | GNAS; GNAS-AS1             | Intron; | Island | 2.44*10⁻⁵ | cg04257105  | -1.31 | 0.52 | 1.25*10⁻² | -2.45  | 1.69 | 0.146     |
|             |      |            |            |         |     |                            | TSS1500 |        |           | cg20528838  | -1.20 | 0.42 | 4.24*10⁻³ | -2.52  | 1.61 | 0.119     |
|             |      |            |            |         |     |                            |         |        |           | cg27661264  | -1.39 | 0.46 | 2.53*10⁻³ | -3.15  | 2.24 | 0.160     |
|             |      |            |            |         |     |                            |         |        |           | cg19589727  | -1.23 | 0.45 | 6.27*10⁻³ | -5.2   | 1.88 | 0.783     |
|             |      |            |            |         |     |                            |         |        |           | cg10302550  | -1.06 | 0.54 | 4.69*10⁻² | -4.54  | 2.31 | 0.049     |
|             |      |            |            |         |     |                            |         |        |           | cg17414107  | -1.32 | 0.59 | 2.54*10⁻² | -1.81  | 2.75 | 0.510     |
| Mineral dust | MN7  | chr17      | 20799408   | 20799694 | 6   | CCDC144NL;                 | TSS200; | Island | 1.13*10⁻⁵ | cg08458692  | -1.06 | 0.54 | 5.03*10⁻² | -6.07  | 2.30 | 0.008     |
|             |      |            |            |         |     | RP11-344E13.3              | 5’UTR   |        |           | cg14560110  | -3.36 | 1.36 | 1.37*10⁻¹ | -9.67  | 6.06 | 0.111     |
|             |      |            |            |         |     |                            |         |        |           | cg08288433  | -3.05 | 0.93 | 1.05*10⁻³ | -8.33  | 7.66 | 0.277     |
|             |      |            |            |         |     |                            |         |        |           | cg06809326  | -2.81 | 0.92 | 2.14*10⁻³ | -10.25 | 5.65 | 0.070     |
|             |      |            |            |         |     |                            |         |        |           | cg22570042  | -3.20 | 1.01 | 1.48*10⁻³ | -14.71 | 7.64 | 0.054     |
|             |      |            |            |         |     |                            |         |        |           | cg21980100  | -2.05 | 0.85 | 1.62*10⁻² | -2.90  | 3.94 | 0.461     |

Chr, Chromosome; CpG, DNA-methylation site; SE, standard error.
Figure 1. Overview of the performed analyses and results per occupational exposure. All analyses were performed for the three exposures in never-smokers.

Table 3. Results of replicated DMRs in never-smokers who were associated with gene expression levels for genes located within 1 MB of the CpG (n = 2802)

| DMR     | CpG     | Annotated gene | Ensembl_ID  | Gene       | B       | SE      | P adjusted |
|---------|---------|----------------|-------------|------------|---------|---------|------------|
| Gases/fumes |         |                |             |            |         |         |            |
| GN13    | cg04257105 | GNAS          | ENSG00000254419 | NPEPL1    | −0.428  | 0.134   | 2.19×10⁻² |
|         | cg17414107 | GNAS          | ENSG00000254419 | NPEPL1    | −0.388  | 0.125   | 2.81×10⁻² |
|         | cg19589727 | GNAS          | ENSG00000254419 | NPEPL1    | −0.580  | 0.188   | 3.03×10⁻² |
|         | cg20528838 | GNAS          | ENSG00000254419 | NPEPL1    | −0.472  | 0.156   | 3.68×10⁻² |
| MN7     | cg06809326 | CCDC144NL     | ENSG00000226981 | ABHD17AP6 | −1.132  | 0.251   | 9.44×10⁻⁵ |
|         | cg08288433 | CCDC144NL     | ENSG00000226981 | ABHD17AP6 | −0.992  | 0.231   | 2.67×10⁻⁴ |
|         | cg14560110 | CCDC144NL     | ENSG00000226981 | ABHD17AP6 | −0.977  | 0.187   | 2.57×10⁻⁶ |
|         | cg21980100 | CCDC144NL     | ENSG00000226981 | ABHD17AP6 | −1.327  | 0.304   | 1.92×10⁻⁴ |
|         | cg22570042 | CCDC144NL     | ENSG00000226981 | ABHD17AP6 | −1.032  | 0.216   | 2.57×10⁻⁵ |

CpG, DNA-methylation site; B, beta; SE, standard error; P adjusted, FDR correct meta-analysis p-value based on genes with available data located within the 1 MB window of the CpG.

and CCDC144NL. CpGs within the DMRs annotated to GNAS and CCDC144NL were significantly associated with lower expression levels of NPEPL1 and ABHD17AP6, respectively. Moreover, 14 out of 21 DMRs were associated with gene expression levels and 11 DMRs were located within the transcript start sites (TSSs) of a gene. Together, our data suggest that occupational exposures may induce differential DNA methylation at specific genomic locations and this may be a mechanism through which occupational exposures affect health.

Interestingly, the majority of identified DMRs were located within the TSS of a gene; 55.2% and 48.5% of the CpGs within the DMRs associated with gases/fumes and mineral dust, respectively, were located in the TSS, compared to 25.7% of all included CpGs in the study. The three replicated DMRs were also located in
Figure 2. Regional association plots (R package comet) for the three replicated DMRs in never-smokers. (A) DMR annotated to VTRNA2–1, (B) DMR annotated to GNAS, and (C) DMR annotated to CCDC144NL. X-axis, megabase (Mb) position on the chromosome; y-axis, negative log10 of the P-values; dots, CpG sites; and see inset legend for the correlation explanation between CpGs.

the TSS, of which two were also associated with gene expression levels (GNAS and CCDC144NL). The general idea of the function of DNA methylation at these TSSs is that it blocks the initiation of transcription and thereby lowers gene expression (9). In the current study, we observed that occupational exposure is associated with lower DNA methylation levels which in turn are associated with higher gene expression levels for most DMRs associated with gene expression levels. This observation thus corroborates our knowledge of the function of DNA methylation at TSSs. Moreover, several of the DMRs associated with gene expression were not associated with the annotated gene. This is partly due to the fact that for 11 of our identified DMRs no gene expression data was available for the annotated gene, including the replicated DMR annotated to VTRNA2–1. For others, CpGs within a DMR were nominally associated with expression levels of the annotated gene but did not survive the multiple testing correction (e.g. the replicated DMR annotated to GNAS).

Another intriguing observation is that several DMRs that we identified are annotated to or associated with the expression of genes with unknown function, RNA genes or pseudo-genes, like CCDC144NL, ABHD17AP6, NPEPL1, RP11-373 N24.2. and LINC02169. It is therefore challenging to understand the relation between these genes and occupational exposures. Long non-coding RNAs (lncRNAs) are known to play a role in gene expression regulation during development, cell differentiation, genomic imprinting and sex chromosomal dosage compensation (16). The gene ZSCAN26 is a zinc finger (transcription factor) and may therefore also be involved in gene expression regulation (17). In addition, multiple microRNAs and lncRNAs were shown to be key regulators of gene expression in lung diseases such as asthma and COPD (18). These might even be biomarkers or therapeutic targets, but more research into the function of these genes is warranted. For your interest, results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) term enrichment analyses are included in Supplementary Material, Tables S7 and S8. Overall, gene expression changes could drive DNA methylation changes due to cellular differentiation as a response to occupational exposures.
However, it is more likely that occupational exposures may affect regulation of gene expression by changing DNA methylation levels of particular genes that regulate the expression of other genes. Interestingly, the three DMRs annotated to RPLP1 and LINCU2169 (2×) were identified in both the gases/fumes and mineral dust analyses. In addition, CpGs annotated to VTRNA2-1 were also associated with occupational exposure to pesticides in our previous study (13). RPLP1 is a ribosomal protein regulating translation and VTRNA2-1 is indirectly also related to the innate immune response, since it was shown to inhibit protein kinase R (EIF2AK2) (19,20). This could indicate that different types of occupational exposures affect similar pathways; alternatively it could result from multiple occupational exposures in specific jobs. For example, construction workers can be exposed to mineral dust and gases/fumes at the same time and crop farmers distribute pesticides over their fields using fuelled machines (gases/fumes exposure). Notably, eight subjects of our cohort were highly exposed to all three occupational exposures and the exposures are moderately to strongly correlated (correlation between gases/fumes and mineral dust = 0.85, between gases/fumes and biological dust = 0.66 and between mineral dust and biological dust = 0.56; Supplementary Material, Table S9). Since we used broad categories of occupational exposures, it was not possible to investigate specific exposure molecules. Occupational exposure levels were also estimated based on current or last held job, and duration of exposure was not taken into account. It is likely that some subjects classified as non-exposed have changed from an ‘exposed’ to a ‘non-exposed’ job, because they experienced adverse effects from the exposures. Therefore, we may have underestimated the effect of occupational exposures on DNA methylation. However, in our cohort on average 72% of the subjects currently exposed to gases/fumes, mineral or biological dust had this job for >5 years and thus had been exposed for a substantial time period in the same job.

Another restriction of our study is the use of blood DNA methylation levels. DNA methylation is cell and tissue specific, and the main route of occupational exposure is via inhalation or skin absorption. However, we have validated a number of CpGs associated with cigarette smoke exposure in lung tissue that were originally identified in whole blood (21). Thus using whole blood could be an efficient way to identify differential DNA methylation upon exposures as an accessible proxy for changes in lung tissue. For your interest, the associations between the lung function measurements FEV₁, FVC, FEV₁/FVC and FEF₂₅₋₇₅ and CpGs within DMRs are provided in Table S10. Furthermore, using a job exposure matrix does not allow to assess specific chemical compounds present in occupational exposures, nor the effect of lifetime exposure. Different types of jobs classified into the same exposure category might contain different chemical compounds as well. Therefore, our results reflect the effect of current or recent occupational exposure on DNA methylation.

In conclusion, our data suggest that occupational exposures may induce differential methylation of genes that regulate gene expression and therefore occupational exposures may induce adverse health effects via this methylation. Several of our identified differentially methylated regions upon occupational exposure to gases/fumes and mineral dust were associated with gene expression levels. Some regions were even associated with two types of occupational exposure. Given the millions of workers that are exposed daily to occupational exposures, further studies on this epigenetic mechanism and health outcomes are warranted. For example, since 40% of the occupational cause of death is due to COPD, especially in developing countries without proper precautions, further studies on this epigenetic mechanism could aid in reducing the global burden of COPD (1).

Materials and Methods

Population and measurements
From the LifeLines Cohort Study, 1656 unrelated subjects were selected for DNA methylation assessment (13). Subject selection was based on creating relatively equal-sized groups based on age, smoking, occupational exposures and spirometry. In the current study only never-smokers were included in order to determine the effects of occupational exposures independent of smoking exposure. No, low and high occupational exposures to gases/fumes, mineral dust and biological dust were estimated using the ALOHA+ job exposure matrix, based on current or last held job (6,22). See Figure 1 and supplementary methods for an overview and more detailed information on the methods.

Genome-wide methylation data and analysis

Illumina Infinium Human Methylation 450 K arrays were used to obtain genome-wide DNA methylation data from whole blood. Samples were processed using the Illumina protocol. Quality control (QC) using Minfi and normalization using DASEN (watermelon) were performed in R (23,24). QC steps included the removal of samples with >1% of all probes having a detection P > 0.01, and samples with incorrect sex or SNP prediction. We removed single probes with a detection P > 0.01, sex chromosome probes, cross-reactive probes (25), probes measuring SNPs and probes where the CpG itself or the single base extension (SBE) site is a SNP. After QC, we had complete data for 420 938 CpG probes in 903 never-smoking subjects.

We performed three genome-wide methylation analyses, one per occupational exposure being gases/fumes, mineral dust and biological dust. We assessed associations between DNA methylation levels (beta-values ranging from 0 to 1) and the three occupational exposures separately using robust linear regression in R (MASS package). Models included low and high exposure dummy-variables (no exposure as reference), and were adjusted for sex, age, technical variances and differential blood counts (eosinophilic, neutrophilic and basophilic granulocytes, lymphocytes and monocytes, all obtained using standard laboratory techniques). Single CpGs with a FDR adjusted P < 0.05 for the high-exposure dummy variable were considered genome-wide significant.

Identification of DMRs

To identify DMRs, comb-p in Python was used (14). Comb-p identifies regions of enrichment by combining adjacent P-values into FDR adjusted regional P-values using auto-correlation and sliding windows. As input we used P-values of the high-exposure dummy variable with the following settings: seed = 0.01 and distance = 300. Each CpG within a DMR with a Sidák-corrected P < 0.05 was further investigated (26).

Validation of DMRs

DMRs identified in LifeLines were validated in the baseline assessment of the third Rotterdam Study cohort (RS-III-1, n = 722) (27). Blood DNA methylation levels were measured using Illumina 450 K arrays and processed similar to LifeLines.
as described elsewhere (28). All CpGs within the DMRs were validated in RS-III-1 and the statistical models (robust linear regression) were similar to LifeLines. Single CpGs and CpGs within the DMRs with a nominal validation P < 0.05 and same direction of effect in both cohorts were considered significantly replicated.

Association between CpGs within DMRs and gene expression
To assess whether the CpGs within the DMRs were associated with gene expression levels, we used data from four population-based cohorts within Biobank-based Integrative Omics Studies (BIOS), from the Biobanking and Biomolecular Resources Research Infrastructure for The Netherlands (BBMRI-NL) (29). In total, 2802 subjects were included in the analyses (independent samples of LifeLines, n = 727; Rotterdam Study III-2, n = 589; Netherlands Twins Registry, n = 900; and Leiden Longevity Study, n = 586) (30-33). In each cohort, probes (read counts from RNA sequencing) within 1 Mb around the CpG were assessed and the linear regression was adjusted for sex, smoking, age and technical variances. Effect estimates of the cohorts were meta-analyzed. CpGs with a meta-analysis FDR-corrected P-value below 0.05 were considered significant.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
The authors wish to acknowledge the services of the LifeLines Cohort Study and the Rotterdam Study, the contributing research centers delivering data to LifeLines and the participating general practitioners in the Rotterdam Study and all the study participants. The authors also thank Mr Michael Verbiest, Ms Mila Jhamai, Ms Sarah Higgins, Mr Marijn Verkerk and Lisette Stolk, PhD, for their help in creating the methylation database.

The LifeLines Biobank initiative has been made possible by funds from FES (Fonds Economische Structuurversterking), SNN (Samenwerkingsverband Noord Nederland) and REP (Ruimtelijke Economisch Programma). The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. The generation and management of the Illumina 450 K methylation array data (EWAS data) for the Rotterdam Study was executed by the Human Genotyping Facility of the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, the Netherlands. The EWAS data was funded by the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, and by the Netherlands Organization for Scientific Research (NWO; project number 184021007) and made available as a Rainbow Project (RP3; BIOS) of the Biobanking and Biomolecular Research Infrastructure Netherlands (BBMRI-NL). The Biobank-Based Integrative Omics Studies (BIOS) Consortium is funded by BBMRI-NL, a research infrastructure financed by the Dutch government (NWO 184.021.007).

Conflict of Interest statement. L.L. reports personal fees from Boehringer Ingelheim GmbH, non-financial support from Novartis, grants from AstraZeneca, grants and non-financial support from European Respiratory Society and grants and non-financial support from Belgian Respiratory Society, outside the submitted work. DSP reports: The University of Groningen has received money for D.S.P regarding a grant for research from Astra Zeneca, Chiesi, Genentec, GSK and Roche. Fees for consultancies were given to the University of Groningen by Astra Zeneca, Boehringer Ingelheim, Chiesi, GSK, Takeda and TEVA. All other authors declare they have no actual or potential competing financial interest.

Funding
Lung Foundation Netherlands (4.1.13.007, 4.113.007 to D.A.v.d.P. and N.A.); Research Foundation—Flanders (postdoctoral fellowship to L.L.).

References
1. Concha-Barrientos, M., Nelson, D.I., Driscoll, T., Steenland, N.K., Punnett, L., Fingerhut, M., Prüss-Üstün, A., Leigh, J., Tak, S.W. and Corvalán, C. (2010) Chapter 21: Selected occupational risk factors. In Comparative Quantification Of Health Risks. World Health Organization, pp. 1651–1801.
2. Iversen, M. and Dahl, R. (2000) Working in swine-confinement buildings causes an accelerated decline in FEV1: a 7-yr follow-up of Danish farmers. Eur. Respir. J., 16, 404–408.
3. Bakke, B., Ulvestad, B., Stewart, P. and Eduard, W. (2004) Cumulative exposure to dust and gases as determinants of lung function decline in tunnel construction workers. Occup. Environ. Med., 61, 262–269.
4. Wang, X., Zhang, H.X., Sun, B.X., Dai, H.L., Hang, J.Q., Eisen, E., Su, L. and Christiani, D.C. (2008) Cross-shift airway responses and long-term decline in FEV1 in cotton textile workers. Am. J. Respir. Crit. Care Med., 177, 316–320.
5. de Jong, K., Boezen, H.M., Kromhout, H., Vermeulen, R., Vonk, J.M., Postma, D.S. and Study, L.C. (2014) Pesticides and other occupational exposures are associated with airway obstruction: the LifeLines cohort study. Occup. Environ. Med., 71, 88–96.
6. Melén, E., Barouki, R., Barry, M., Boezen, H.M., Hoffmann, B., Kraus-Etschmann, S., Koppelman, G.H. and Forsberg, B. (2018) Promoting respiratory public health through epigenetics research: an ERS environment health committee workshop report. Eur. Respir. J., 51.
7. Ruiz-Hernandez, A., Kuo, C.C., Rentero-Garrido, P., Tang, W.Y., Redon, J., Ordovas, J.M., Navas-Acien, A. and Tellez-Plaza, M. (2015) Environmental chemicals and DNA methylation in adults: a systematic review of the epidemiologic evidence. Clin. Epigenetics, 7, eCollection 2015.
8. Jones, P.A. (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. Natl. Rev., 13, 484–492.
9. Hussain, M.B., Valter, M., Concha, G. and Broberg, K. (2012) Low-level environmental cadmium exposure is associated with DNA hypomethylation in Argentinean women. Environ. Health Perspect., 120, 879–884.
11. Li, C., Yang, X., Xu, M., Zhang, J. and Sun, N. (2013) Epigenetic marker (LINE-1 promoter) methylation level was associated with occupational lead exposure. Clin. Toxicol. (Phila.), 51, 225–229.
12. Goodrich, J.M., Basu, N., Franzblau, A. and Dolinoy, D.C. (2013) Mercury biomarkers and DNA methylation among Michigan dental professionals. Environ. Mol. Mutagen., 54, 195–203.
13. van der Plaat, D.A., de Jong, K., de Vries, M., van Diemen, C.C., Nedeljković, I., Amin, N., Kromhout, H., Biobank-based Integrative Omics Study Consortium, Vermeulen, R., Postma, D.S. et al. (2018) Occupational exposure to pesticides is associated with differential DNA methylation. Occup. Environ. Med., 75, 427–435.
14. Pedersen, B.S., Schwartz, D.A., Yang, I.V. and Kechris, K.J. (2012) Comb-p: software for combining, analyzing, grouping and correcting spatially correlated P-values. Bioinformatics, 28, 2986–2988.
15. Zeilinger, S., Kuhnel, B., Klopp, N., Baurecht, H., Kleinschmidt, A., Gieger, C., Weidinger, S., Lattka, E., Adameski, J., Peters, A. et al. (2013) Tobacco smoking leads to extensive genome-wide changes in DNA methylation. PLoS One, 8, e63812.
16. Fatica, A. and Bozzoni, I. (2014) Long non-coding RNAs: new players in cell differentiation and development. Natl. Rev., 15, 7–21.
17. Klug, A. (2010) The discovery of zinc fingers and their applications in gene regulation and genome manipulation. Annu. Rev. Biochem., 79, 213–231.
18. Booton, R. and Lindsay, M.A. (2014) Emerging role of MicroRNAs and long noncoding RNAs in respiratory disease. Chest, 146, 193–204.
19. Martinez-Azorin, F., Remacha, M. and Ballesta, J.P. (2008) Functional characterization of ribosomal P1/P2 proteins in human cells. Biochem. J., 413, 527–534.
20. Kunkeaw, N., Jeon, S.H., Lee, K., Johnson, B.H., Tanasanvimon, S., Javle, M., Pairojkul, C., Chamgramol, Y., Wongfieng, W., Gong, B. et al. (2013) Cell death/proliferation roles for nc886, a non-coding RNA, in the protein kinase R pathway in cholangiocarcinoma. Oncogene, 32, 3722–3731.
21. de Vries, M., van der Plaat, D.A., Nedeljkovic, I., Verkaik-Schakel, R.N., Kooistra, W., Amin, N., van Duijn, C.M., Brandsma, C.-A., van Diemen, C.C., Vonk, J.M. et al. (2018) From blood to lung tissue: effect of cigarette smoke on DNA methylation and lung function. Respir. Med., 19, 212.
22. Matheson, M.C., Benke, G., Raven, J., Sim, M.R., Kromhout, H., Vermeulen, R., Johns, D.P., Walters, E.H. and Abramson, M.J. (2005) Biological dust exposure in the workplace is a risk factor for chronic obstructive pulmonary disease. Thorax, 60, 645–651.
23. Aryee, M.J., Jaffe, A.E., Corrada-Bravo, H., Ladd-Acosta, C., Feinberg, A.P., Hansen, K.D. and Irizarry, R.A. (2014) Minfi: a flexible and comprehensive bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics, 30, 1363–1369.
24. Pidsley, R., Y Wong, C.C., Volta, M., Lunnon, K., Mill, J. and Schalkwyk, L.C. (2013) A data-driven approach to preprocessing Illumina 450K methylation array data. BMC Genomics, 14, 293.
25. Chen, Y.A., Lemire, M., Choufani, S., Butcher, D.T., Grafodatskaya, D., Zanke, B.W., Gallinger, S., Hudson, T.J. and Weksberg, R. (2013) Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics, 8, 203–209.
26. Sidak, Z. (1967) Rectangular confidence regions for the means of multivariate Normal distributions. J. Am. Stat. Assoc., 62, 626–633.
27. Hofman, A., Brusselle, G.G., Darwish Murad, S., van Duijn, C.M., Franco, O.H., Goedegebure, A., Ikram, M.A., Klaver, C.C., Nijsten, T.E., Peeters, R.P. et al. (2015) The Rotterdam Study: 2016 objectives and design update. Eur. J. Epidemiol., 30, 661–708.
28. Ligthart, S., Steenaard, R.V., Peters, M.J., van Meurs, J.B., Sijbrands, E.J., Uitterlinden, A.G., Banden, M.J., Consortium, B., Hofman, A., Franco, O.H. et al. (2016) Tobacco smoking is associated with DNA methylation of diabetes susceptibility genes. Diabetologia, 59, 998–1006.
29. Biobanking and Biomolecular Resources Research Infrastructure for The Netherlands (BBMRI-NL) (2017) Biobank-based integrative Omics studies (BIOS). 2017. Available: https://www.bbmri.nl/acquisition-use-analyze/bios.
30. Tighelaar, E.F., Zernakova, A., Dekens, J.A., Hermes, G., Baranska, A., Mujagic, Z., Swertz, M.A., Munoz, A.M., Deelen, P., Cenit, M.C.C. et al. (2015) Cohort profile: Lifelines DEEP, a prospective, general population cohort study in the northern Netherlands: study design and baseline characteristics. BMJ Open, 5, e006772-e002014-006772.
31. Hofman, A., van Duijn, C.M., Franco, O.H., Ikram, M.A., Janssen, H.L., Klaver, C.C., Kuipers, E.J., Nijsten, T.E., Stricker, B.H., Tiemeier, H. et al. (2011) The Rotterdam Study: 2012 objectives and design update. Eur. J. Epidemiol., 26, 657–686.
32. Willemsen, G., Vink, J.M., Abellanou, A., den Braber, A., van Beek, J.H., Draisma, H.H., van Dongen, J., van’t Ent, D., Geels, L.M., van Lien, R. et al. (2013) The adult Netherlands twin register: twenty-five years of survey and biological data collection. Twin Res. Hum. Genet., 16, 271–281.
33. Westendorp, R.G., van Heemst, D., Krome, M., Mooijjstra, S.P., Blauw, G.J., Beekman, M., Heijmans, B.T., de Craen, A.J., Slagboom, P.E. et al. (2009) Nonagenarian siblings and their offspring display lower risk of mortality and morbidity than sporadic nonagenarians: the Leiden longevity Study. J. Am. Geriatr. Soc., 57, 1634–1637.