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

ABSTRACT

Background: Many workers are daily exposed to occupational agents like gases/fumes, mineral dust or biological dust, but how these exposures induce adverse health effects is still largely unknown. 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.

Methods: To determine the effects of occupational exposures independent of smoking, 903 never-smokers of the Dutch LifeLines cohort study were included. Illumina 450K arrays were used to obtain genome-wide blood DNA methylation data. We performed three genome-wide methylation analyses, 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 phython. Results were validated in the Rotterdam Study (233 never-smokers) and methylation-expression associations were assessed using Biobank-based Integrative Omics Studies Consortium (BIOS) data (n=2,802).

Results: Of in total 21 significant DMRs, 14 DMRs were associated with gases/fumes and 7 with mineral dust. Three DMRs were associated with both gases/fumes and mineral dust (*RPLP1* and *LINC02169* (2x)). The majority of DMRs were located within transcript start sites of gene expression regulating genes (11 out of 21 DMRs) and some DMRs were located in genes that were previously linked to lung diseases. We replicated three DMRs, two with gases/fumes (*VTRNA2-1* and *GNAS*), and one with mineral dust (*CCDC144NL*). Fifteen DMRs, 9 with gases/fumes and 6 with mineral dust, significantly associated with gene expression levels.

Conclusion: 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.

Keywords: Occupational exposure; DNA methylation; Epigenetic epidemiology; Genome-wide

INTRODUCTION

Daily, millions of workers worldwide are exposed to chemical agents, fumes, and (in) organic dusts.(WHO 2010) The leading occupational causes of death in 2000 were unintentional injuries (41%), chronic obstructive pulmonary disease (COPD, 40%) and lung cancer (13%).(WHO 2010) 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 (FEV_1).(Bakke et al. 2004; Iversen and Dahl 2000; Wang et al. 2008) In addition, we have previously shown that exposure to gases/fumes, mineral and biological dust is associated with small and large airways obstruction.(de Jong et al. 2014a, 2014b)

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.(Melén et al. 2018) Environmental exposures, like occupational exposures, induce changes in DNA methylation levels, which can affect gene expression, possibly aiding in disease development.(Ruiz-Hernandez et al. 2015) 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.(Jones 2012) Several small studies showed suggestive evidence that specific compounds found in occupational exposures, like cadmium, lead, and mercury affect DNA methylation.(Goodrich et al. 2013; Hossain et al. 2012; Li et al. 2013; Ruiz-Hernandez et al. 2015)

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.

METHODS

Population and measurements

From the Lifelines Cohort Study, 1,656 unrelated subjects were selected for DNA methylation assessment.(van der Plaat et al. 2018) 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 exposure to gases/fumes, mineral dust and biological dust was estimated using the ALOHA+ job exposure matrix (JEM), based on current or last held job.(de Jong et al. 2014b; Matheson et al. 2005) See Figure 1 and supplementary methods for an overview and more detailed information on the methods.

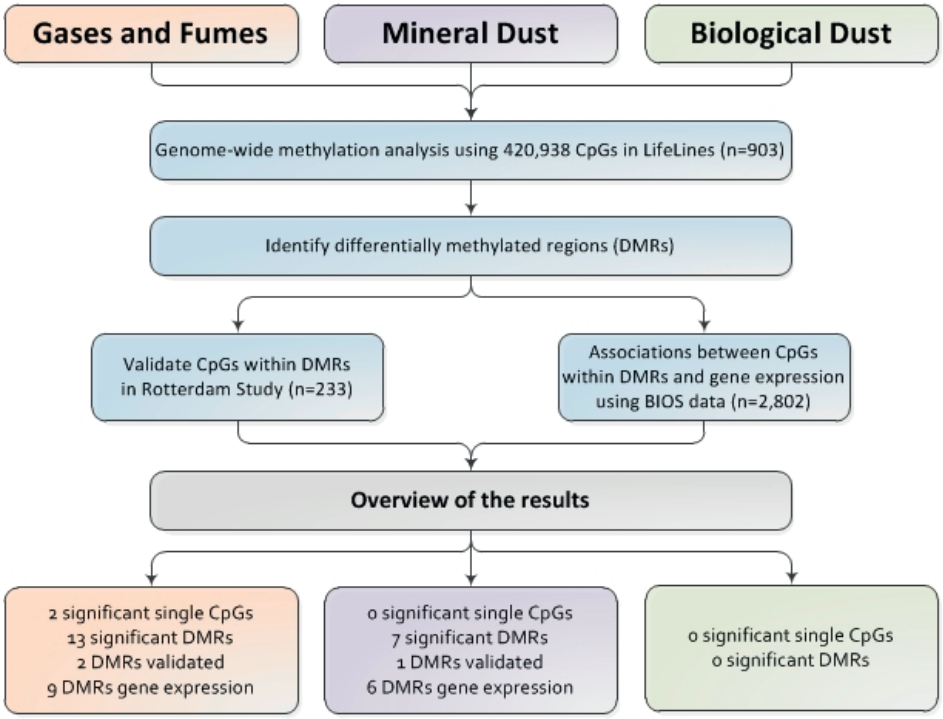

Figure 1: Overview of the performed analyses and results per occupational exposure. All analyses were performed for the three exposures in never-smokers.

Genome-wide methylation data and analysis

Illumina Infinium Human Methylation 450K arrays were used to obtain genome-wide blood DNA methylation data. Samples were processed using the Illumina protocol. Quality control (QC) using *Minfi* and normalization using *DASEN* (*watermelon*) was

performed in R.(Aryee et al. 2014; Pidsley et al. 2013) Quality-control (QC) steps included the removal of samples with >1% of all probes having a detection p-value >0.01, and samples with incorrect sex or SNP prediction. We removed single probes with a detection p-value >0.01, sex chromosome probes, cross-reactive probes(Chen et al. 2013), 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 false discovery rate (FDR) adjusted p-value <0.05 for the high exposure dummy-variable were considered genome-wide significant.

Identification of differentially methylated regions (DMRs)

To identify differentially methylated regions (DMRs), *comb-p* in python was used. (Pedersen et al. 2012) 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 Šidák corrected p-value <0.05 was further investigated.(Sidak 1967)

Validation of DMRs

DMRs identified in Lifelines were validated in the baseline assessment of the third Rotterdam Study cohort (RS-III-1, n=722).(Hofman et al. 2015) Blood DNA methylation levels were measured using Illumina 450K arrays and processed similar to LifeLines as described elsewhere.(Ligthart et al. 2016) 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-value <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).(BBMRI-NL. 2017) In total, 2,802 subjects were included in the analyses (independent samples of LifeLines (n=727), Rotterdam Study III-2 (n=589), Netherlands Twin Registry (n=900), and Leiden Longevity Study (n=586)).(Hofman et al. 2011; Tigchelaar et al. 2015; Westendorp et al. 2009; Willemsen et al. 2013) In each cohort, probesets (read counts from RNA sequencing) within 1Mb 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-analysed. CpGs with a meta-analysis p-value below the Bonferroni corrected threshold (p=0.05/number of probesets in 1MB window) were considered significant.

RESULTS

Population characteristics

Complete data on all covariates were available for 903 never-smokers in the identification cohort LifeLines and 233 never-smokers in the validation cohort Rotterdam Study (Table 1 and Table S1).

We present the results of our analyses per exposure (i.e. gases/fumes, mineral dust and biological dust). For an overview of all results see Figure 1. The results of all analyses can be found in the supplementary Excel-file and the Manhattan plots are shown in Figure S1.

Table 1. Characteristics of the never-smokers included in the LifeLines cohort study (discovery cohort) and the Rotterdam Study (validation cohort).

	LifeLines	**Rotterdam Study**
N with no missing data	903	233
Males, N (%)	508 (57)	100 (43)
Age (years), median (min-max)	46 (18-80)	57 (47-89)
Occupational exposure, N	*No/Low/High*	*No/Low/High*
Gases/Fumes	637 / 150 / 116	177 / 51 / 5
Mineral dust	673 / 105 / 125	210 / 20 / 3
Biological dust	720 / 69 / 114	N/A

SD = Standard deviation N/A = Not applicable
See table S1 for the characteristics of the LifeLines cohort separately per exposure level.
NB The LifeLines sample is a selected population, not a sample from the general population

Gases/Fumes

Genome-wide methylation analysis

In the genome-wide methylation analysis in never-smokers, two single CpGs were epigenome-wide significantly associated with gases/fumes exposure (FDR<0.05) (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 (Table S3). The three most significant DMRs are annotated to long intergenic non-protein coding RNA 2169 (LINC02169), Ribosomal Protein Lateral Stalk Subunit P1 (RPLP1) and leptin (LEP). The genome-wide significant CpG annotated to RPL37A was not located within an identified DMR.

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 (Table 2 and Table 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*) (Figure 2 A/B).

Gene expression analysis

We found that CpGs within nine 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 gene expression results of all DMRs identified with gases/fumes see Table S5. The replicated DMRs annotated to *GNAS* was 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 (FDR<0.05), but seven DMRs were (Tables 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.

Table 2. Results of the CpGs within the replicated differentially methylated regions (DMRs) associated with occupational exposures in never-smokers.

				N	Annotation			P		LifeLines			Rotterdam Study		
DMR	**Chr**	**Start**	**End**	**probes**	**Gene**	**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	$1.18*10^{-2}$	-2.33	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; TSS1500	Island	$2.44*10^{-2}$	cg04257105	-1.31	0.52	$1.25*10^{-2}$	-2.45	1.69	0.146
									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	$6.27*10^{-3}$	-.52	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; RP11-344E13.3*	TSS200; 5'UTR	Island	$1.13*10^{-3}$	cg08458692	-1.06	0.54	$5.03*10^{-2}$	-6.07	2.30	**0.008**
									cg14560110	-3.36	1.36	$1.37*10^{-2}$	-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

DMR = Differentially methylated region Chr = Chromosome CpG = DNA-methylation site P = p-value SE = Standard Error

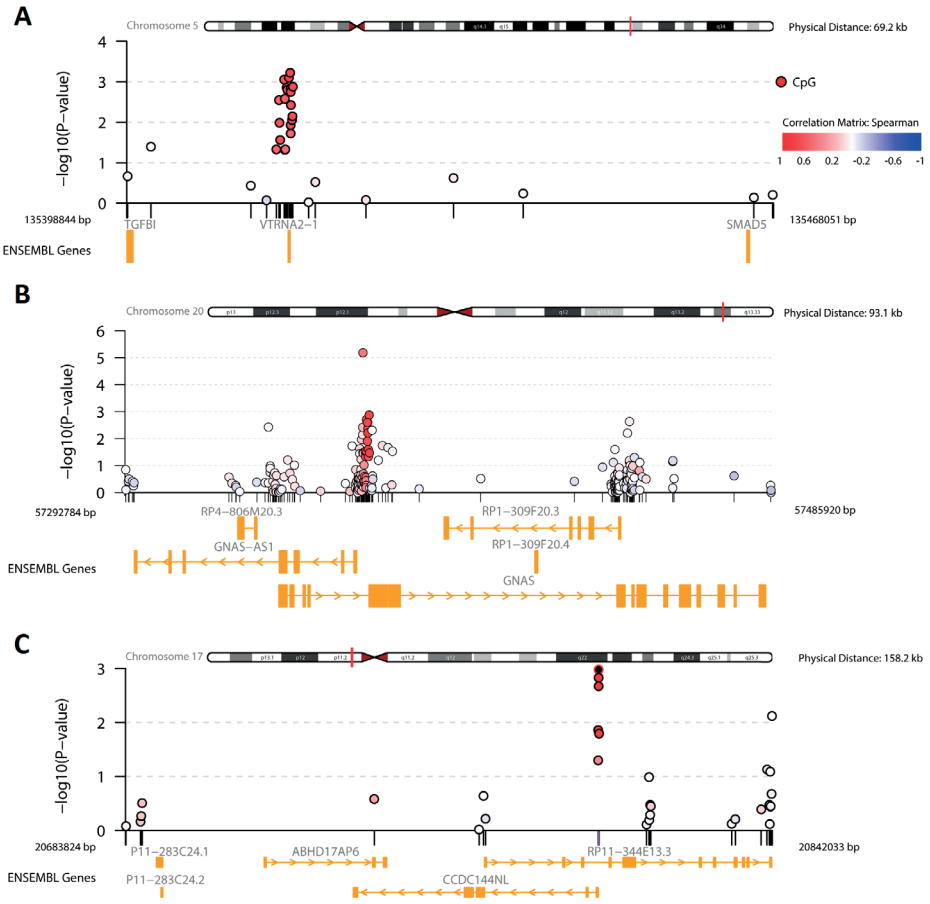

Figure 2: Regional association plots (R package comet) for the three replicated DMRs in never-smokers. A) DMR annotated to *VTRNA2-1*, B) DMR annotated to *GNAS*, and C) DMR annotated to *CCDC144NL*. x-axis = megabase (Mb) position on the chromosome, y-axis = negative log10 of the p-values, dots = CpG-sites, and see inset legend for the correlation explanation between CpGs.

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 (Tables 2 and S4, and Figure 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

(Table S6). The replicated DMR annotated to *CCDC144NL* was associated with lower expression of Abhydrolase Domain Containing 17A Pseudogene 6 (*ABHD17AP6*).

Biological dust

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

Table 3. Results of replicated DMRs in never-smokers which were associated with gene expression levels for genes located within 1MB of the CpG (n=2,802)

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	$3.67*10^{-2}$
Mineral dust							
MN7	cg06809326	*CCDC144NL*	ENSG00000226981	*ABHD17AP6*	-1.132	0.251	$9.44*10^{-5}$
	cg08288433	*CCDC144NL*	ENSG00000226981	*ABHD17AP6*	-0.992	0.231	$2.68*10^{-4}$
	cg14560110	*CCDC144NL*	ENSG00000226981	*ABHD17AP6*	-0.977	0.187	$2.57*10^{-6}$
			ENSG00000170298	*LGALS9B*	0.554	0.168	$1.42*10^{-2}$
	cg21980100	*CCDC144NL*	ENSG00000226981	*ABHD17AP6*	-1.327	0.304	$1.92*10^{-4}$
	cg22570042	*CCDC144NL*	ENSG00000226981	*ABHD17AP6*	-1.032	0.216	$2.56*10^{-5}$

DMR = Differentially methylated region CpG = DNA-methylation site B = beta SE = Standard Error P adjusted = Bonferroni correct meta-analysis p-value based on genes with available data located within the 1MB window of the CpG.

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.(Zeilinger et al. 2013) In these never-smokers, occupational exposure to gases/fumes and to mineral dust was associated with 14 and 7 differentially methylated regions (DMRs), respectively. Three of these DMRs were associated with both gases/fumes and mineral dust (1 DMR in *RPLP1* and 2 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* 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 (TSS) 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, including the three replicated DMRs, 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.(Jones 2012) In the current study, we observed that occupational exposure is associated with lower DNA methylation levels which in turn is 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 *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 pseudogenes, like *CCDC144NL, ABHD17AP6, NPEPL1, RP11-373N24.2,* 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.(Fatica and Bozzoni 2014) The gene *ZSCAN26* is a zinc finger (transcription factor) and may therefore also be involved in gene expression regulation.(Klug 2010) In addition, multiple microRNAs and lncRNAs were shown to be key regulators of gene expression in lung diseases such as asthma and COPD.(Booton and Lindsay 2014) These might even be biomarkers or therapeutic targets, but more research into the function of these genes is warranted. Overall, our data seem to suggest that occupational exposures 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 *LINC02169* (2x) 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.(van der Plaat et al. 2018) *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*).(Kunkeaw et al. 2013; Martinez-Azorin et al. 2008) 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, 8 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, Table S7). 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 either gases/fumes, mineral or biological dust had this job for more than 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.[submitted] 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. Furthermore, using a JEM 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 suggests 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.(WHO 2010)

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