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Occupational exposure to pesticides is associated with differential DNA methylation

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ABSTRACT

Objectives Occupational pesticide exposure is associated with a wide range of diseases, including lung diseases, but it is largely unknown how pesticides influence airway disease pathogenesis. A potential mechanism might be through epigenetic mechanisms, like DNA methylation. Therefore, we assessed associations between occupational exposure to pesticides and genome-wide DNA methylation sites.

Methods 1561 subjects of LifeLines were included with either no (n=1392), low (n=108) or high (n=61) exposure to any type of pesticides (estimated based on current or last held job). Blood DNA methylation levels were measured using Illumina 450K arrays. Associations between pesticide exposure and 420 938 methylation sites (CpGs) were assessed using robust linear regression adjusted for appropriate confounders. In addition, we performed genome-wide stratified and interaction analyses by gender, smoking and airway obstruction status, and assessed associations between gene expression and methylation for genome-wide significant CpGs (n=2802).

Results In total for all analyses, high pesticide exposure was genome-wide significantly (false discovery rate P<0.05) associated with differential DNA methylation of 31 CpGs annotated to 29 genes. Twenty of these CpGs were found in subjects with airway obstruction. Several of the identified genes, for example, *RYR1*, *ALLC*, *PTPRN2*, *LRRC3B*, *PAX2* and *VTRNA2-1*, are genes previously linked to either pesticide exposure or lung-related diseases. Seven out of 31 CpGs were associated with gene expression levels.

Conclusions We show for the first time that occupational exposure to pesticides is genome-wide associated with differential DNA methylation. Further research should reveal whether this differential methylation plays a role in the airway disease pathogenesis induced by pesticides.

Key messages

What is already known about this subject?
- Millions of workers worldwide are exposed daily to occupational pesticide exposure, but it is largely unknown how pesticides influence airway disease pathogenesis.
- To date, no large-scale epigenome-wide association study assessing the association between DNA methylation and occupational pesticide exposures has been performed.

What are the new findings?
- Occupational pesticide exposure is associated with differential DNA methylation, and this may be an important mechanism how pesticides induce airway diseases.
- We identified multiple differentially methylated sites associated with pesticide exposure, and some of these were associated with gene expression levels as well.

How might this impact on policy or clinical practice in the foreseeable future?
- Further research should reveal whether this differential methylation plays a role in the disease pathogenesis induced by pesticides and potential preventive interventions.

INTRODUCTION

Pesticides, including insecticides, herbicides and fungicides, are widely used in the agricultural sector to protect crops against harmful or unwanted insects, weeds and fungi. Exposure to pesticides can, however, also be toxic to humans and it has been estimated that approximately 250 000 people worldwide die of pesticide poisoning each year.[1] In the occupational setting, it has been estimated that about 25 million workers experience unintentional pesticide poisoning each year, due to inhalation or skin absorption.[2] Long-term exposure to pesticides, like in occupational settings, has been associated with an increased risk for birth defects, several types of cancer and chronic diseases such as Parkinson's disease, diabetes, chronic obstructive pulmonary disease (COPD), atherosclerosis and autoimmune diseases.[3–5] Moreover, our own previous studies have shown that occupational exposure to pesticides is associated with lower lung function levels and airway obstruction cross-sectionally and with accelerated lung function decline longitudinally.[6 7]

Despite the associations with a wide range of diseases and health outcomes, it is still largely unknown how pesticides affect disease development. Several mechanisms have been proposed that may underlie the detrimental effects of pesticide

exposures, such as induction of oxidative stress, disruption of the endocrine system, mitochondrial dysfunction and epigenetic modifications.[4] A well-known epigenetic modification is DNA methylation, which is the binding of a methyl group to a cytosine base adjacent to a guanine base (a CpG) site.[8] DNA methylation can alter gene expression without changing the DNA sequence and is increasingly recognised as an important link between environmental exposures and disease.[9] Altered DNA methylation levels have been found to play a role in multiple complex diseases, such as cancer, respiratory and neurodegenerative diseases.[10] An in vitro study by Zhang *et al* provided evidence that gene promoter DNA methylation levels are indeed altered upon exposure to pesticides.[11] Furthermore, studies in Greenlandic Inuit as well as in Koreans have shown that exposure to persistent organic pollutants is associated with global hypomethylation, based on the *Alu* and LINE-1 assay.[12–14] These studies thus showed that pesticide exposure affects global DNA methylation levels, and therefore it might be possible that differential methylation at specific genomic locations may contribute to the deleterious effects of pesticides.

The current study is the first large-scale epigenome-wide association study assessing associations between occupational exposure to pesticides and DNA methylation levels. Since pesticide exposure can affect organs in the entire body, we used blood methylation to assess the effects of occupational exposure to any type of pesticide on DNA methylation levels.[4] In addition, since there might be interindividual differences in the effects of pesticides on methylation, we decided to stratify our analysis based on susceptibility groups. We stratified by gender because previous research observed gender differences in pesticide-related health effects.[15 16] In our previous studies, we have shown that the association between pesticide exposure and lung function is dependent on smoking habits, and therefore we assessed if the association between pesticide exposure and DNA methylation differs between never-smokers and current-smokers.[6 7] Furthermore, since the main route of pesticide exposure is via inhalation and occupational exposure to pesticides is associated with airway obstruction,[6] we assessed if DNA methylation on pesticide exposure differs between subjects with and without airway obstruction. Finally, we assessed whether the identified differentially methylated sites are associated with gene expression levels in blood.

METHODS

Population and measurements

In total, 1656 subjects were selected of the Dutch population-based cohort study LifeLines at baseline (2006–2011).[17] All subjects provided written informed consent and the study was approved by the Medical Ethics Committee of the University Medical Centre Groningen, Groningen, the Netherlands. Subjects were specifically selected from the larger cohort taking smoking history (never-smoker or current-smoker), airway obstruction (defined as forced expiratory volume in 1 s to forced vital capacity ratio (FEV_1/FVC) <70%) and occupational related exposures into account. To optimise the exposure contrast, self-reported never-smokers with 0 pack-years of smoking and current-smokers with >5 pack-years were selected. Occupational exposure to pesticides was estimated based on current or last held job using the ALOHA+ Job Exposure Matrix (JEM), which classifies subjects based on the ISCO-88 job codes into no (0), low (1) and high (2) exposure categories, as published previously.[6 18 19]

To assess whether methylation levels at the identified CpGs are associated with gene expression levels in blood, we used data of the BIOS (Biobank-based Integrative Omics Studies) project, from the Biobanking and Biomolecular Resources Research Infrastructure for The Netherlands (BBMRI-NL).[20] In total, 2802 subjects were selected from four population-based cohorts, LifeLines (n=727)[21] Rotterdam Study III-2 (n=589),[22] Netherlands Twin Registry (n=900)[23] and Leiden Longevity Study (n=586).[24]

Genome-wide methylation assay

The Illumina Infinium Human Methylation 450K arrays (Illumina, San Diego, California, USA) were used to determine genome-wide DNA methylation levels at approximately 485 000 CpG sites of blood taken at the baseline visit. We randomised 1656 LifeLines subjects based on sex, exposure and airway obstruction across the arrays. Using 500 ng DNA for each sample, we first performed a bisulphite conversion using the EZ-96 DNA methylation kit (Zymo Research, Irvine, California, USA), which was validated using commercially available bisulphite conversion control samples (Zymo Research). After this step, the samples were processed according to the Illumina 450K protocol. After quality control, the final data set contained data for 1561 subjects and 420 938 CpG probes (see online supplementary methods for quality control).

Statistical analysis

To assess the relationship between occupational exposure to pesticides and DNA methylation levels, we used robust linear regression models in R (MASS package), which are less sensitive to outliers and heteroscedastic errors compared with linear least-squares models.[25] Beta values were used to represent DNA methylation levels, which is the ratio between the intensities of methylated versus unmethylated probes, ranging from 0 to 1.

We included two dummy variables in the model for occupational pesticide exposure, that is, low and high, no exposure being the reference. To estimate possible batch effects, a principal component (PC) analysis was performed using the control probes included on the 450K chip.[26] We included seven PCs in the final model that each explained >1% of the variance. Together, these seven PCs captured 95.5% of the total variance. Interestingly, the position on the chip was an important determinant of the measured variance (online supplementary methods). In addition, the model was adjusted for differential blood counts for lymphocytes, monocytes and eosinophilic, neutrophilic and basophilic granulocytes obtained using standard laboratory techniques. The final model was adjusted for sex, age, current-smoking, pack-years, batch effects (PCs) and differential blood counts. When applicable, the model was adjusted for the single nucleotide polymorphism (SNP) under the probe since it can influence the accuracy of the assay.

We analysed the association between pesticide exposure and DNA methylation in the complete cohort and stratified by gender (men/women), smoking (never/current) or by airway obstruction. In addition, we assessed interactions between pesticide exposure and gender, current-smoking or airway obstruction on genome-wide methylation levels. For all analyses, CpG sites with false discovery rate (FDR) adjusted P value <0.05 for the high pesticide exposure variable were considered genome-wide significant.

Finally, in each of the four population-based cohorts of the BIOS dataset, we assessed the association between methylation and gene expression for the identified CpGs. The linear

Table 1 Characteristics of the subjects included in LifeLines

	Complete cohort	Men	Women	Never- smokers	Current- smokers	Subjects without airway obstruction	Subjects with airway obstruction
n without missing data	1561	883	678	903	658	966	595
Men, n (%)	883 (57)	883 (100)	0 (0)	508 (56)	375 (57)	588 (61)	295 (50)
Age (years), median (min–max)	46 (18–80)	46 (19–79)	46 (18–80)	46 (18–80)	46 (22–79)	44 (18–79)	49 (26–80)
Pesticide exposures, n (%)							
No	1392 (89)	762 (83)	630 (93)	786 (87)	606 (92)	836 (87)	556 (93)
Low	108 (7)	76 (9)	32 (5)	72 (8)	36 (6)	81 (8)	27 (5)
High	61 (4)	45 (5)	16 (2)	45 (5)	16 (2)	49 (5)	12 (2)
Current-smokers	658 (42)	375 (43)	283 (41)	0 (0)	658 (100)	379 (39)	279 (47)
Pack-years, mean (min–max)	8.7 (0–100)	9 (0–100)	8 (0–58)	0	21 (5–100)	7 (0–68)	11 (0–100)
Pulmonary function, mean (SD)							
FEV_1/FVC (%)	73 (9)	74 (9)	73 (8.48)	74 (8)	72 (9)	79 (5)	64 (5)
FEV_1%predicted (%)*	92 (14)	93 (14)	91 (14)	95 (14)	89 (14)	97 (12)	84 (14)
Airway obstruction, n (%)†	595 (38)	295 (33)	300 (44)	316 (35)	279 (42)	0 (0)	595 (100)
GOLD COPD stage ≥2, n (%)‡	158 (10)	83 (9)	75 (11)	56 (6)	102 (16)	0 (0)	158 (26)
Differential blood counts, mean (SD)							
Basophilic granulocytes (%)	0.5 (0.3)	0.5 (0.30)	0.5 (0.4)	0.6 (0.3)	0.5 (0.4)	0.5 (0.3)	0.6 (0.3)
Eosinophil granulocytes (%)	3.1 (1.9)	3.2 (1.93)	3.0 (1.9)	3.1 (2.1)	3.1 (1.7)	3.1 (1.9)	3.2 (2.0)
Neutrophil granulocytes (%)	55.0 (8.45)	54.4 (8.13)	55.8 (8.6)	54.5 (8.2)	55.8 (8.5)	55.2 (8.3)	54.8 (8.5)
Lymphocytes (%)	33.2 (7.5)	33.3 (7.32)	33.1 (7.8)	33.7 (7.5)	32.5 (7.5)	33.1 (7.6)	33.4 (7.5)
Mononuclear cells (%)	8.1 (1.9)	8.6 (1.91)	7.6 (1.8)	8.2 (1.9)	8.1 (2.0)	8.2 (1.9)	8.1 (1.9)

*FEV_1%predicted is based on the reference equation by Global Lung Function Initiative 2012 (GLI-2012).
†FEV_1/FVC <70%.
‡Global Initiative for Chronic Obstructive Lung Disease (GOLD) COPD stage: FEV_1/FVC <70% and FEV_1%predicted <80%.
COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

regression was adjusted for sex, smoking, age and batch effects (cohort-specific PCs). All genes with expression data available within 1 Mb around the CpG were assessed, and the results of the cohorts were meta-analysed based on the effect estimates (random-effect model). CpGs with a meta-analysis P value below the Bonferroni-corrected threshold (P≤0.05/number of probe-sets in the 1 Mb window) were considered significant.

Additional analyses

In the online supplementary methods, additional analyses related to the association of pesticide exposure with differential blood cell counts, PCs or age acceleration based on the epigenetic age are shown. In addition, results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) term enrichment analyses are also included in the online supplementary methods.

RESULTS

Population characteristics

Complete data on all covariates were available for 1561 subjects of the LifeLines cohort. Of the included subjects, 883 were men and 678 were women; 903 were never-smokers and 658 were current-smokers; 595 subjects had airway obstruction and 966 subjects did not (table 1). For an overview of all analyses, see online supplemental figure 1.

Complete cohort

In the complete cohort, CpGs were not significantly associated with high exposure to pesticides on a genome-wide significant level (FDR<0.05).

Stratification by gender

In men, no CpGs were significantly associated with high exposure to pesticides on a genome-wide level (FDR<0.05).

In women, high exposure to pesticides was significantly associated with higher levels of DNA methylation at four CpGs (table 2). These CpGs are annotated to LY6/PLAUR Domain Containing 6 (*LYPD6*), ATP Synthase, H+Transporting, Mitochondrial Fo Complex Subunit C3 (*ATP5G3*), Sodium/Potassium Transporting ATPase Interacting 3 (*NKAIN3*) and TBC1 Domain Family Member 9 (*TBC1D9*). A fifth CpG (cg23116540) showed a lower DNA methylation level upon pesticide exposure and annotated to Thrombospondin1 (*THBS1*). For two CpGs (*ATP5G3* and *THBS1*), the effect estimate for the low exposure variable was in the same direction as the high exposure variable, but not significant. The CpG annotated to *ATP5G3* was associated with higher expression levels of this gene (table 3).

Interaction between pesticide exposure and gender

The CpG annotated to *NKAIN3* was significantly higher methylated on pesticide exposure in women compared with men (table 4).

Stratification by never-smoking and current-smoking

In never-smokers, one CpG (cg03181524) was genome-wide significantly associated with high exposure to pesticides. This CpG was annotated to Ryanodine receptor 1 (*RYR1*), named after the natural plant-derived insecticide ryanodine. This CpG was higher methylated in both low and high exposed subjects, and the P value of the low exposure variable was borderline significant (table 2 and figure 1A). The CpG was associated with lower expression levels of three genes, namely *RYR1,* Calpain 12 (*CAPN12*) and *CTD-2540F13.2* (table 3).

Table 2 Genome-wide significant associations between DNA methylation and exposure to pesticides stratified by gender, smoking status and by the presence of airway obstruction

								Low pesticide exposure			High pesticide exposure		
CpG	Chr	Bp	Str	Gene	Island	Position	Mean beta (%)	B	SE	P	B	SE	P
Women (n=678)													
cg10770187	2	150 186 923	–	LYPD6	Island	Intron; TSS200	5.90	−0.15	0.29	0.603	2.37	0.41	7.69×10^{-9}
cg16884400	2	176 046 185	–	ATP5G3	Island	5′ UTR	6.41	0.01	0.23	0.953	1.74	0.32	4.10×10^{-8}
cg08855288	8	63 161 589	–	NKAIN3	Island	5′ UTR	6.53	−0.32	0.23	0.169	1.69	0.32	1.47×10^{-7}
cg23116540	15	39 871 808	–	THBS1	N_Shore	TSS1500	85.85	−0.43	0.50	0.386	−3.55	0.69	2.77×10^{-7}
cg04256347	4	141 677 674	+	TBC1D9	Island	TSS1500	5.82	−0.24	0.17	0.160	1.23	0.24	4.04×10^{-7}
Never-smokers (n=903)													
cg03181524	19	39 048 014	+	RYR1	N_Shelf	Body	35.3	1.39	0.77	0.070	6.32	0.96	5.20×10^{-11}
Current-smokers (n=658)													
cg07158503	5	135 415 693	–	VTRNA2-1	N_Shore	TSS1500	46.6	0.60	1.73	0.731	−13.20	2.55	2.24×10^{-7}
cg14677130	5	38 445 916	–	EGFLAM	OpenSea	Body	85.8	0.64	0.91	0.485	−6.91	1.34	2.73×10^{-7}
Subjects with airway obstruction (n=595)													
cg15793258	19	4 638 503	–	TNFAIP8L1	N_Shore	TSS1500	61.1	0.15	1.22	0.904	−20.78	1.82	3.30×10^{-30}
cg25251562	2	3 704 773	–	ALLC	OpenSea	TSS1500	84.0	−2.75	1.08	0.011	−11.65	1.62	5.91×10^{-13}
cg27484412	21	44 876 687	+	LINC00319	Island	3′ 3 kb	9.3	−0.28	0.26	0.281	2.12	0.39	4.17×10^{-8}
cg15577272	7	157 932 397	+	PTPRN2	OpenSea	Body	82.5	−0.24	0.61	0.699	−4.87	0.91	9.31×10^{-8}
cg12839813	16	89 114 720	–	CTD-2555A7.2	Island	Body	51.7	−0.92	1.02	0.367	8.07	1.53	1.25×10^{-7}
cg10059942	10	124 908 488	+	HMX2	Island	Body	10.2	−0.24	0.37	0.517	2.80	0.55	2.91×10^{-7}
cg17623116	3	26 751 066	+	LRRC3B	OpenSea	5′ UTR	83.7	−0.06	0.27	0.821	−1.98	0.40	8.84×10^{-7}
cg20869844	7	140 103 726	–	RAB19	OpenSea	TSS200	9.2	0.04	0.31	0.900	2.20	0.46	1.29×10^{-6}
cg12425700	4	75 186 779	–	BC016361	OpenSea	Body	77.4	−0.07	0.37	0.847	−2.64	0.55	1.46×10^{-6}
cg26867890	6	39 692 938	+	KIF6	Island	Body	6.3	0.16	0.14	0.262	1.03	0.21	1.53×10^{-6}
cg09961397	6	70 991 205	–	COL9A1	N_Shore	Body	79.6	−1.78	0.77	0.021	−5.51	1.15	1.55×10^{-6}
cg03943218	10	102 419 600	+	PAX2	Island	5′ 85 kb	10.5	−0.18	0.27	0.509	1.90	0.40	2.11×10^{-6}
cg20800022*	8	97 169 764	+	GDF6	Island	Body	12.8	0.32	0.31	0.312	−0.70	0.15	2.12×10^{-6}
cg11465769	2	1 800 785	–	MYT1L	N_Shore	Body	77.4	−0.26	0.49	0.599	−3.46	0.73	2.18×10^{-6}
cg10645314	2	3 704 589	–	ALLC	OpenSea	TSS1500	83.5	−5.23	1.71	0.002	−12.05	2.55	2.21×10^{-6}
cg19084031	15	38 361 362	–	RP11-1008C21.2	N_Shelf	3′ 7 kb	18.3	0.09	0.70	0.904	4.97	1.05	2.23×10^{-6}
cg04035638	6	31 141 768	+	PSORS1C3; POU5F1	OpenSea	Body (exon); body	85.3	−0.12	0.31	0.706	−2.19	0.47	2.51×10^{-6}
cg19611616*	12	27 397 833	–	STK38L	S_Shore	5′ UTR	20.2	0.25	0.34	0.474	0.01	0.10	2.62×10^{-6}
cg13082120	6	33 257 050	+	WDR46; PFDN6	S_Shore	1st exon; TSS1500	11.5	0.49	0.36	0.179	2.55	0.54	2.70×10^{-6}
cg07508480*	2	46 496 100	+	EPAS1	OpenSea	5′ 28 kb	88.8	−0.02	0.24	0.934	−0.32	0.12	2.90×10^{-6}

*Additionally adjusted for single nucleotide polymorphism (SNP) under the probe.
There were no significant associations between DNA methylation and pesticide exposure in the full cohort and in the stratified analysis in men or in individuals without airway obstruction.
B, beta; Bp, base pair; Ch, chromosome; CpG, DNA methylation site; Str, strand, UTR, untranslated region; TSS, transcription start site.

Table 3 Significant associations between DNA methylation and gene expression (n=2802)

CpG	Annotated gene	Ensembl_ID	Gene	B	SE	P adjusted
Women						
cg16884400	*ATP5G3*	ENSG00000154518	*ATP5G3*	0.098	0.005	2.94×10^{-2}
Never-smokers						
cg03181524	*RYR1*	ENSG00000196218	*RYR1*	−0.881	0.157	6.74×10^{-7}
		ENSG00000182472	*CAPN12*	−0.877	0.194	1.86×10^{-4}
		ENSG00000267892	*CTD-2540F13.2*	*−0.916*	0.234	2.76×10^{-3}
Subjects with airway obstruction						
cg10059942	*HMX2*	ENSG00000154473	*BUB3*	1.062	0.378	3.45×10^{-2}
		ENSG00000138161	*CUZD1*	1.474	0.536	4.18×10^{-2}
cg20869844	*RAB19*	ENSG00000146955	*RAB19*	−3.479	1.134	2.37×10^{-2}
		ENSG00000157764	*BRAF*	−0.724	0.248	3.90×10^{-2}
cg11465769	*MYT1L*	ENSG00000130508	*PXDN*	4.973	1.445	2.32×10^{-3}
cg19084031*	*RP11-1008C21.2*	ENSG00000259225	*RP11-1008C21.1*	−1.711	0.177	2.34×10^{-21}
		ENSG00000166068	*SPRED1*	−0.513	0.086	1.18×10^{-8}
cg04035638	*PSORS1C3;POU5F1*	ENSG00000225851	*HLA-S*	10.888	1.621	7.57×10^{-10}
		ENSG00000204580	*DDR1*	1.678	0.328	1.25×10^{-5}
		ENSG00000137310	*TCF19*	1.239	0.345	1.33×10^{-2}
		ENSG00000228789	*HCG22*	−5.066	1.421	1.48×10^{-2}
		ENSG00000234745	*HLA-B*	−1.337	0.389	2.46×10^{-2}

No data in BIOS-BBMRI were available for cg08855288, cg15793258 and cg27484412. In online supplemental table 5, the results of all CpGs and genes with expression data available within 1 Mb are shown.
*CpG also identified in pesticide exposure×airway obstruction analysis.
B, beta; CpG, DNA methylation site; P adjusted, Bonferroni-corrected P values based on all probesets located within the 1 Mb window.

In current-smokers, high exposure to pesticides was significantly associated with lower levels of DNA methylation at two CpGs (table 2). These CpGs are annotated to (vault) RNA gene *VTRNA2-1* (also known as *MIR886*, figure 1B) and EGF Like, Fibronectin Type III And Laminin G Domains (*EGFLAM*). For both CpGs, the effect estimate of the low exposure variable was in the opposite direction (higher methylation) and not significant. The identified CpGs were not significantly associated with gene expression levels.

Interaction between pesticide exposure and current-smoking

Four CpGs had a genome-wide significant interaction with high pesticide exposure and current-smoking and three of these are annotated to *VTRNA2-1* (figure 1B). Current-smokers who are highly exposed to pesticide exposure have lower DNA methylation levels at these *VTRNA2-1* loci compared with exposed never-smokers (table 4). The other CpG is located in an intron of Elongator Acetyltransferase Complex Subunit 4 (*ELP4*). None of these four CpG sites were associated with gene expression levels.

Stratification according to airway obstruction

In subjects without airway obstruction, no CpGs were significantly associated with high exposure to pesticides on a genome-wide level (FDR<0.05).

In subjects with airway obstruction (FEV_1/FVC<70%), 20 CpGs were significantly associated with high exposure to pesticides (table 2). Nine CpGs had higher levels of DNA methylation on pesticide exposure and are annotated to the long intergenic non-protein coding RNA 391 (*LINC000319*), *AK055272*, H6 Family Homeobox 2 (*HMX2*), *RAB19*, Kinesin Family Member 6 (*KIF6*), Paired Box 2 (*PAX2*), *LOC101928227*, Serine/Threonine Kinase 38 Like (*STK38L*) and WD Repeat Domain 46 (*WDR46*)/Prefoldin Subunit 6 (*PFDN6*). The other 11 CpGs had lower DNA methylation levels on pesticide exposure and annotated to Tumour Necrosis Factor, Alpha-Induced Protein 8-Like 1 (*TNFAIP8L1*, figure 1C), Allantoicase (*ALLC*, two CpGs, figure 1D), Protein Tyrosine Phosphatase Receptor Type N2 (*PTPRN2*), Leucine Rich Repeat Containing 3B (*LRRC3B*), *BC016361*, Collagen Type IX Alpha 1 Chain (*COL9A1*), Growth Differentiation Factor 6 (*GDF6*), Myelin Transcription Factor 1 Like (*MYT1L*), Psoriasis Susceptibility 1 Candidate 3 (*PSORS1C3*) and Endothelial PAS Domain Protein 1 (*EPAS1*). For 11 CpGs, the effect estimate for the low exposure variable was in the same direction as the high exposure variable, and for the three CpGs annotated to *ALLC* (2x) and *COL9A1*, the effect estimate for the low exposure variable was significant. The CpGs annotated to *RAB19* and *RP11-1008C21.2* were significantly associated with lower gene expression levels in blood (table 3) and the CpGs annotated to *HMX2* and *MYT1L* were associated with higher gene expression levels. The CpG annotated to *PSORS1C3* was associated with higher gene expression levels of *HLA-S*, *DDR1* and *TCF19*, and with lower expression of *HCG22* and *HLA-B*.

Interaction between pesticide exposure and airway obstruction

Three CpGs annotated to *TNFAIP8L1*, *ALLC* and *LRRC3B* were significantly lower methylated on pesticide exposure in subjects with airway obstruction compared with those without airway obstruction (table 4). In addition, two CpGs were higher methylated on pesticide exposure in subjects with airway obstruction compared with subjects without airway obstruction, and are located in the body of the RNA gene *CTD-2555A7.2* (*AK055272*) and *LOC101928227*. None of these CpGs were associated with gene expression levels.

Additional information

The Manhattan, volcano and Q–Q plots of all analyses are shown in online supplemental figures 1–11 and the regional

Table 4 Genome-wide significant interactions between pesticide exposure and gender, current-smoking or airway obstruction on DNA methylation levels

CpG	Chr	Bp	Str	Gene	Island	Position	Mean beta (%)	High pesticide exposure			Sex/current-smoking/airway obstruction			Interaction		
								B	SE	P	B	SE	P	B	SE	P
Pesticide exposure×sex (n=1561)																
cg08855288	8	63 161 589	–	*NKAIN3*	Island	5′ UTR	6.53	−2.39	0.52	4.59×10^{-6}	−0.28	0.21	0.184	2.06	0.39	1.01×10^{-7}
Pesticide exposure×current-smoking (n=1561)																
cg07158503	5	135 415 693	–	*VTRNA2-1*	N_Shore	TSS1500	47.4	0.14	1.09	0.900	−0.51	0.65	0.428	−15.95	2.10	3.23×10^{-14}
cg06478886	5	135 416 029	–	*VTRNA2-1*	N_Shore	TSS200	36.7	0.32	0.89	0.718	−0.20	0.52	0.700	−10.42	1.70	9.64×10^{-10}
cg10994681	11	31 672 091	+	*ELP4*	Body	Body	86.2	−1.31	0.36	0.0003	−0.08	0.21	0.712	3.64	0.70	1.92×10^{-7}
cg11608150	5	135 415 948	–	*VTRNA2-1*	N_Shore	TSS1500	30.8	0.30	1.12	0.788	−0.92	0.67	0.167	−11.24	2.16	2.02×10^{-7}
Pesticide exposure×airway obstruction (n=1561)																
cg15793258	19	4 638 503	–	*TNFAIP8L1*	N_Shore	TSS1500	62.6	−0.59	0.76	0.440	−0.57	0.30	0.058	−20.19	1.69	6.19×10^{-33}
cg25251562	2	3 704 773	–	*ALLC*	OpenSea	TSS1500	83.7	0.78	0.89	0.377	0.58	0.35	0.093	−12.20	1.96	4.72×10^{-10}
cg12839813	16	89 114 720	–	*CTD-2555A7.2*	Island	Body	50.9	−0.61	0.75	0.418	0.56	0.29	0.057	9.00	1.66	5.55×10^{-8}
cg17623116	3	26 751 066	+	*LRRC3B*	OpenSea	5′ UTR	83.6	0.57	0.52	0.007	0.15	0.08	0.064	−2.43	0.46	1.61×10^{-7}
cg19084031	15	38 361 362	–	*RP11-1008C21.2*	N_Shelf	3′ 7kb	18.5	−0.83	0.00	0.109	0.02	0.20	0.935	5.69	1.15	6.72×10^{-7}

B, beta; Bp, base pair; Chr, chromosome; CpG, DNA methylation site; Str, strand, UTR, untranslated region , TSS, transcription start site .

epigenome-wide association plots of most identified loci (coMET plots)[27] are shown in online supplemental figures 12–17. In addition, online supplemental tables 1–4 show the associations of all CpGs identified in all performed analyses and online supplemental table 5 shows the associations between all CpGs and genes with available expression data within 1 Mb of CpG.

The results of the additional analyses showed that high pesticide exposure was not significantly associated with any of the blood cell types or age acceleration based on the epigenetic age (online supplementary methods). Moreover, in women, five GO terms were significant (FDR<0.05) and these are homophilic cell adhesion via plasma membrane adhesion molecules, cell–cell signalling, cell–cell adhesion via plasma membrane adhesion molecules, calcium ion binding and plasma membrane part.

DISCUSSION

To our knowledge, this is the first study to assess the association between occupational exposure to pesticides and genome-wide DNA methylation levels. There were no significant associations between pesticide exposure and DNA methylation in the complete population. However, we identified a total of 31 CpG sites, annotated to 29 genes, that were associated with high pesticide exposure in the stratified analyses or the interaction analyses based on gender, smoking or airway obstruction status. Of these 31 CpGs, 5 CpGs were genome-wide significant in women, of which one CpG annotated to *NKAIN3* was significantly different between men and women. In addition, one CpG annotated to *RYR1* was identified in never-smokers and two CpGs annotated to *VTRNA2-1* and *EGFLAM* were identified in current-smokers. In the interaction analysis between smoking and pesticide exposure, four CpGs were genome-wide significant, of which three CpGs were annotated to *VTRNA2-1* and one was also identified in the current-smokers. Lastly, 20 of these 31 CpGs were genome-wide significantly associated with high pesticide exposure in subjects with airway obstruction. The associations of these 20 sites were not significant in subjects without airway obstruction, and in addition, 5 of these 20 sites were genome-wide significant in the interaction analysis between the presence of airway obstruction and pesticide exposure. Our data therefore suggest that differential methylation at specific genomic locations as induced by pesticides may play a role in airway disease pathogenesis.

Some of the annotated genes of the 20 CpGs identified in subjects with airway obstruction were previously associated with lung function or lung diseases, like *ALLC*, *PTPRN2*, *LRRC3B* and *PAX2*. Moreover, genetic variants in the *ALLC* gene were previously associated with changes in FEV_1 following inhaled corticosteroid treatment.[28] Hypermethylation of *PTPRN2* was seen in squamous cell lung cancer samples, and the gene's methylation profile is included in prediction models for a COPD, pulmonary fibrosis and lung cancer diagnosis.[29 30] *LRRC3B* was differentially methylated in several cancer types and suggested to be a tumour suppressor gene in non-small-cell lung cancer.[31] The *PAX2* gene is of interest since *PAX2* is abnormally higher expressed in Foxp1/2/4-deficient developing lungs, is a biomarker for lung cancer and is lower expressed in zebra fish exposed to glyphosate-based herbicides.[32–34] Unfortunately, no expression data were available for *ALLC* and *LRRC3B*, and the association of cg15577272 with *PTPRN2* gene expression was non-significant. However, the association between cg03943218 and *PAX2* expression levels was nominal significant (B=3.29, SE 1.20, P=0.006, online supplemental table S4). Five other CpGs were significantly associated with gene expression levels

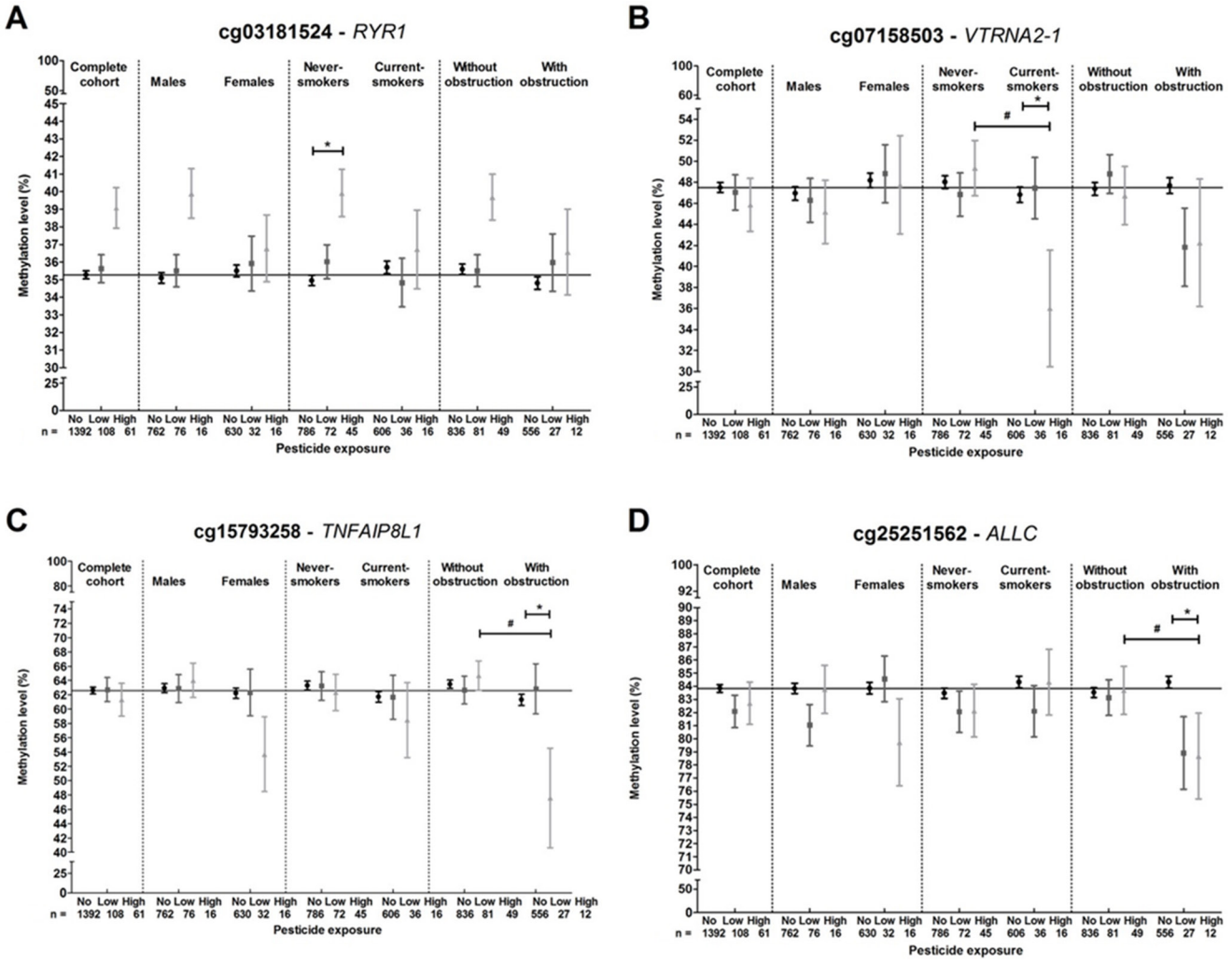

Figure 1 Plots of mean unadjusted methylation levels for no, low and high exposure to pesticides in the complete cohort and stratified by gender, smoking and airway obstruction. (A) cg03181524 annotated to *RYR1*, (B) cg07158503 annotated to *VTRNA2-1*, (C) cg15793258 annotated to *TNFAIP8L1*, (D) cg25251562 annotated to *ALLC*. *Genome-wide significant (false discovery rate corrected P value <0.05). #Genome-wide significant interaction.

(table 3). Interestingly, CpG cg04035638 is located within the *HLA* superlocus and was associated with expression levels of multiple genes within this region, including *HLA-B*, *HLA-S* and *DDR1*. The *HLA* region plays an important role in the immune response and has been associated with asthma and lung cancer.[35] Therefore, differential DNA methylation on exposure to pesticides may alter gene expression levels and subsequently play a role in the development of airway diseases.

Another interesting finding of this study is the observation of higher DNA methylation of a CpG located in an intron of *RYR1* with high exposure to pesticides. Higher DNA methylation at this CpG was associated with lower *RYR1* expression levels. In addition, the observed association between pesticide exposure and higher DNA methylation levels at the *RYR1* intron was only significant in never-smokers, but a similar trend was seen in the complete cohort, in men and in subjects without airway obstruction (figure 1A). The *RYR1* gene codes for a skeletal muscle calcium release channel that can be targeted by anthranilic diamide insecticides.[36] In the current study, it is not possible to assess the effects of specific pesticides, like the anthranilic diamide insecticide ryanodine. Moreover, this is a relatively new class of insecticides introduced around 2006.[37] Since our data collection started in 2006, it is therefore u nknown if the subjects were exposed to this type of insecticide. Our findings do, however, indicate that higher methylation on pesticide exposure is associated with lower *RYR1* expression levels, and it could therefore be a biological plausible mechanism through which pesticides act, but experimental studies are warranted.

In women, we also identified possible interesting differently methylated CpGs on pesticide exposure in, among others, the transcription start sites (TSSs) of the genes *THBS1* and *LYPD6*. Alterations in the expression of the transcription factor *THBS1* were found on in vitro exposure of human peripheral blood lymphocytes to the pesticides cypermethrin and mancozeb.[38] The *LYPD6* protein was found to directly interact with the nicotinic acetylcholine receptor in brain extracts, which is also a target for several classes of insecticides.[39 40] Although we were not able to assess the effects of these specific pesticides, these findings do point to plausible genes related to pesticide exposure.

We also identified three CpGs located in the TSS of the vault RNA *VTRNA2-1* (or *MIR886*) that are lower methylated with high pesticide exposure in current-smokers compared with never-smokers (figure 1B). This vault RNA is located between Transforming Growth Factor Beta 1 (*TGFB1*) and SMAD Family Member 5 (*SMAD5*), and both genes have been implicated in COPD development.[41] Gene expression data of *VTRNA2-1* were

unfortunately not available in the used database, but the CpGs were not associated with the expression of *TGFB1* or *SMAD5*. *VTRNA2-1* was previously found to inhibit Protein Kinase R (EIF2AK2), which plays a role in the innate immune response to viral infection.[42] Interestingly, it has been shown that pesticide exposure can have an immunosuppressive effect in eastern box turtles and frogs.[43 44] It is also known that smokers have an increased risk for both bacterial and viral infections.[45] Treppendahl *et al* showed that when the *VTRNA2-1* locus is lower methylated, as seen in our study as well, *VTRNA2-1* expression is higher, and in turn inhibiting Protein Kinase R.[46] Subjects might therefore be more susceptible to viral infections. This makes it an interesting locus to investigate in future studies in relation to pesticide (and smoking) exposure.

Interestingly, when low pesticide exposure was nominal (borderline) significantly associated with methylation levels of the identified CpGs, the effect estimate of high pesticide exposure was in the same direction, but higher. This suggests a dose–response effect for these CpGs, including CpGs annotated to *RYR1* and *ALLC*. For most of the identified CpGs, however, low pesticide exposures were not significantly associated with methylation levels and in some case the effect estimate was in the opposite direction. This suggests that a high dose of pesticide exposure is needed to affect the DNA methylation level.

To our knowledge, we are the first to assess the association between occupational exposure to pesticides and genome-wide blood DNA methylation levels. Being the first also implied, unfortunately, that we were unable to find a validation cohort with 450K methylation data and enough highly pesticide-exposed subjects to replicate our results. Further research should reveal whether these identified CpGs are true findings, but they do point to a plausible biological mechanism. Another potential limitation of our study is the use of blood DNA methylation levels to assess the association of lung diseases with high exposure to pesticides. We used blood DNA methylation since it is easily accessible and pesticides have systemic effects. However, DNA methylation is cell and tissue specific, thus the effects could be different if we would assess DNA methylation in different tissues. It might be interesting to repeat this study using lung or skin tissue since these are most directly exposed to pesticides. Finally, in the current study, we used the ALOHA+ JEM to estimate the level of occupational pesticide exposure at baseline based on the current or last held job. Previous studies have shown that using a JEM is less likely to be affected by recall bias and differential misclassification compared with self-reported exposure.[47–49] However, heterogeneity within jobs could have caused an underestimation of the effect. Furthermore, it was not feasible in our large population-based study to assess the effect of specific chemical compounds found in pesticides, nor the effect of lifetime pesticide exposure. However, in our cohort, the subjects have been exposed for a substantial time period in the same job since 75% of the subjects currently exposed to pesticides has held the same job for more than 10 years. Our results thus reflect the effect of current or recent occupational exposure to a broad range of pesticides on DNA methylation, but future (experimental) studies on for example organochlorine, organophosphate or carbamate pesticides could provide different (specific) pathological pathways.

CONCLUSIONS

We showed that occupational exposure to pesticides is associated with differential DNA methylation of specific sites in the genome and more particularly in the subgroup with airway obstruction. Several of the annotated genes are biologically plausible genes previously linked to either pesticide exposure or lung-related diseases. Of the 31 differentially methylated CpGs, 7 CpGs were associated with gene expression levels and thus might have a biological function. Further research should reveal whether these identified CpGs are true findings and whether they play a role in the pathogenesis of airway diseases.

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Contributors DAvdP participated in the study design, analysis and interpretation of the data, and drafting of the manuscript, tables and figures. HMB, DSP, CCvD and CMVD obtained funding. JMV, KdJ, HMB, DSP, CCvD, CMVD, MdV, IN and NA determined the study design, participated in the analysis and interpretation of data, and critically supervised writing of the manuscript. HK and RV designed and provided the ALOHA+ JEM. All researchers part of the group author BBMRI BIOS participated in collecting the BIOS data. All authors approved the final version of the manuscript.

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Data sharing statement LifeLines data are available (at costs) to all scientists. Scientists can apply for access to Lifelines data and samples by submitting a research proposal to the LifeLines biobank (www.lifelines.nl). Data on occupational exposures in LifeLines can be obtained from Professor H M Boezen. Access to the Biobank-based Integrative Omics Study (BIOS) data is available by application to the BIOS Data Access Committee (www.bbmri.nl/acquisition-use-analyze/bios/).

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