Epigenome-wide association study on diffusing capacity of the lung: replication and meta-analysis

ABSTRACT

Background: Epigenetics may play an important role in pathogenesis of lung diseases. However, little is known about the epigenetic factors that influence impaired gas exchange at the lungs.

Aim: To identify the epigenetic signatures of the diffusing capacity of the lung measured by carbon monoxide uptake.

Methods: Epigenome association study (EWAS) was performed on diffusing capacity, measured by carbon monoxide uptake (DLCO) and per alveolar volume (DLCO/VA) using the single-breath technique in 2,674 individuals from two population-based cohort studies, the Rotterdam Study (the discovery panel) and the Framingham Heart Study (the replication panel). We assessed the clinical relevance of our findings by investigating the identified sites in whole blood and lung tissue specific gene expression. Finally, we performed an exploratory epigenome-wide analysis, combining both cohorts.

Results: We identified and replicated two CpG sites (cg05575921 and cg05951221) that were significantly associated with DLCO/VA and one (cg05575921) suggestively associated with DLCO. Furthermore, we found a positive association between *AHRR* (cg05575921) hypomethylation and gene expression of *EXOC3* in whole blood. We confirmed that the expression of *EXOC3* is positively associated with DLCO/VA and DLCO in lung tissue. Finally, several genome-wide associations were identified after meta-analysing the data of both cohorts.

Conclusions: We report on epigenome-wide associations with diffusing capacity in the general population. Our results suggest EXOC3 to be an excellent candidate through which smoking induced hypomethylation of *AHRR* might affect pulmonary gas exchange.

INTRODUCTION

Tests for diffusing capacity of the lung for carbon monoxide (DLCO and DLCO per alveolar volume (DLCO/VA)) provide a quantitative measure of gas exchange in the lung. In addition to its utility in the diagnosis and monitoring of lung diseases such as emphysema and pulmonary fibrosis, measure of gas exchange is an independent predictor of mortality in COPD patients (1). DLCO and DLCO/VA measurements are influenced by environmental factors (2). Exposure to environmental factors such as smoking, occupation related compounds and air pollution, decreases the level of gas exchange in the lungs.

DLCO and DLCO/VA are also heritable traits, and genetic variation contributes to the variation of these lung function tests (Terzikhan et al). Although genetics play an important role, epigenetic mechanisms such as DNA methylation are important for regulation of gene expression but also essential to understand the interplay between genes, environment and disease (3). Like DLCO and DLCO/VA, DNA methylation is also influenced by smoking (4, 5); however, the question remains whether DNA methylation is associated with gas exchange independently of smoking.

Little is known about the effects of DNA methylation on lung function (6-8) and epigenome wide association studies (EWAS) of pulmonary gas exchange are lacking. Therefore, we aimed to perform an EWAS to investigate which epigenetic variants are related to the phenotypic variation in DLCO and DLCO/VA. In addition, we examined the impact of the discovered epigenetic variants on gene expression. Finally, we combined the data of both cohorts in an exploratory epigenome-wide analysis.

METHODS

Study populations

This EWAS study encompassed a discovery study embedded within the Rotterdam Study and a replication study embedded within the Framingham Heart Study. The Rotterdam Study is an ongoing prospective population-based cohort study in Rotterdam, the Netherlands. The design has been previously described. Briefly, the Rotterdam Study includes four sub-cohorts. For this study, data from the third visit of the second sub-cohort (RSII-3) and the second visit of the third sub-cohort (RSIII-2) was used. The discovery panel consisted of 650 participants from a random subset of 747 individuals of European descent with methylation and diffusing capacity data available.

Replication of the identified CpG sites was performed in 2,114 individuals form the Framingham Heart Study Offspring cohort from which methylation and diffusing capacity data was available. The design of the Framingham Heart Study has been

described extensively before (9). The replication analyses were focused on Offspring cohort participants of European descent who attended the eighth exam (2005-2008). We only included participants over 45 years of age, with methylation and with blood cell counts data available.

DNA methylation

DNA was extracted from whole peripheral blood (stored in EDTA tubes) by standardised salting out methods. Genome-wide DNA methylation levels were measured using the Illumina Human Methylation 450K array (Illumina, San Diego, CA, USA). In summary, samples (500 ng of DNA per sample) were first treated with bisulfite using the Zymo EZ-96 DNA-methylation kit (Zymo Research, Irvine, CA, USA). Subsequently, samples were hybridised to the arrays according to the standardized protocols. The methylation percentage of a CpG site was presented as a β value ranging between 0 (no methylation) to 1 (full methylation). Data processing was performed in the Genetic Laboratory of Internal Medicine, Erasmus University Medical Centre, Rotterdam. Quality control was performed using Genome Studio (v2011.1, methylation module version 1.9.0; Illumina, USA).

For each probe, individuals with methylation levels higher than three times the inter-quartile range (IQR) were excluded. Finally, we excluded cross reactive and polymorphic probes, or probes that have an underlying SNP at the CpG site, in addition to probes within 10 bp of the single base extension (minor allele frequency >1% in European ancestry (EUR) 1000 genomes project data) (10). In total, 363,387 CpGs were included.

Diffusing capacity of the lung: DLCO and DLCO/VA

DLCO (mmol/min/kPA) and alveolar volume (VA) in liter were measured by the single breath technique in accordance with ERS / ATS guidelines (11). The DLCO per alveolar volume (DLCO/VA; mmol/min/kPA/liter) was calculated by dividing the DLCO by VA. Analyses were restricted to participants with two interpretable and reproducible measurements of DLCO and DLCO/VA. Two measurements were considered reproducible if the difference between the first and second DLCO measurement ((highest-lowest value)/highest value) was ≤10% and the difference between the first and second DLCO/VA measurement was ≤15%.

Covariates

Covariates were selected based on known association with DNA methylation and diffusing capacity and included age, sex, smoking status, weight, height and batch effects; array number and position.

Functional analysis

We used data from the BBMRI atlas (12) (**see URLs**) to identify methylation-gene expression associations; the so called expression quantitative trait methylation (eQTM).

Gene Expression in lung tissue

Lung resection specimens were obtained from 92 patients, of which 78 from surgery for solitary pulmonary tumours (Ghent University Hospital, Ghent, Belgium) and 14 from explant lungs of end-stage COPD patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium). Lung tissue at maximum distance from the pulmonary lesions and without signs of retro-obstructive pneumonia or tumour invasion, was collected by a pathologist. None of the patients operated for malignancy were treated with neo-adjuvant chemotherapy. Written informed consent was obtained from all subjects. This study was approved by the medical ethical committees of the Ghent University Hospital (2011/14) and the University Hospital Gasthuisberg Leuven (S51577).

RNA was extracted with the miRNeasy Mini kit (Qiagen) from total lung tissue blocks submersed in RNA-later. cDNA was obtained by the miScript II RT kit (Qiagen), following manufacturer's instructions. Expression of target genes ADGRG6 (GPR126) and reference genes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine phosphoribosyltransferase-1 (HPRT-1) and Succinate Dehydrogenase Complex Flavoprotein Subunit A (SDHA) were analyzed using Taqman Gene Expression Assays (Applied Biosystems, Forster City, CA, USA). Real-time PCR reactions were set up in duplicate using diluted cDNA using identical amplification conditions for each of the target and reference genes. A standard curve derived from serial dilutions of a mixture of all samples were included in each run. The amplification conditions consisted of: 10 minutes at 95°C and 60 cycles of 95°C for 10 seconds and 60°C for 15 seconds. Amplifications were performed using a LightCycler 96 detection system (Roche). Data were processed using the standard curve method. Expression of target genes was corrected by a normalization factor that was calculated based on the expression of three reference genes, using the geNorm applet according to the guidelines and theoretical framework previously described (13).

Statistical analysis

Methylation probes were tested for association with DLCO or DLCO/VA using a linear regression model in the Rotterdam Study and linear mixed model in the Framingham Heart Study. Surrogate variable analysis was used to adjust for inflation in the effect estimates by batch effect in the Framingham Heart Study. The first model (Model 1) included age, sex, smoking, white blood cells, array number and position. In the second model (Model 2), we additionally adjusted for weight and height. False discovery rate

of 5% was used to correct for multiple testing (FDR < 0.05 was considered statistically significant). Inverse variance weighted meta-analysis was performed using METAL (14) (see URL). We test the association between mRNA expression of *AHRR* and *EXOC3* genes and gas exchange in a linear regression adjusting for all covariates used in Model 2. For these analyses, outliers (mean ±3 SD) were excluded and mRNA levels were log transformed to make sure the normality assumption was fulfilled. A p-value lower than 0.05 was considered statistically significant.

RESULTS

General characteristics

The discovery set consisted of 659 participants from the Rotterdam Study. Replication was performed in a set of 2,114 participants from the Framingham Heart Study. The general characteristics of the study populations are shown in **Table 1**. The mean age (SD) was 67.4 (5.9) in the Rotterdam Study and 65.6 (8.4) in the Framingham Heart Study. Mean levels of DLCO and DLOC/VA were similar in both study populations.

Table 1 General characteristics of the discovery and validation study populations

	RS		FHS	
N total	659		2,114	
Age (yrs), mean (SD)	67.4	(5.90)	65.6	(8.38)
Female, N (%)	369	(56%)	1159	(55%)
Current smokers, N (%)	70	(11%)	151	(7%)
Past smokers, N (%)	379	(58%)	1,186	(56%)
Weight (kg), mean (SD)	79.9	(13.94)	79.5	(17.83)
Height (cm), mean (SD)	169.8	(9.16)	167.3	(9.54)
DLCOc (mmol/min/kPA), mean (SD)	7.81	(1.64)	7.47	(2.13)
DLCOc/VA (mmol/min/kPA/VA), mean (SD)	1.52	(0.23)	1.42	(0.23)

DLCO: Diffusing capacity of the lung for carbon monoxide; DLCO/VA: Diffusing capacity of the lung for carbon monoxide by alveolar volume; FHS: The Framingham Heart Study; RS: The Rotterdam Study.
Values are means (standard deviation (SD)) for continuous variables or counts (percentages %) for dichotomous variables.

Discovery and replication

Epigenomewide association study (EWAS) of DLCO

No statistically significant associations were found between DNA methylation and DLCO in model 1 adjusted for age, sex, smoking status, white blood cell counts and

batch effects, nor in model 2 additionally adjusted for weight and height. However, cg05575921 (Gene: *AHRR*, chromosome: 5) was suggestively associated with DLCO in both models (FDR_{model1}=0.07, FDR_{model2}= 0.09).

EWAS of DLCO/VA

In model 1, we identified one CpG site associated with DLCO/VA; cg05575921 (Gene: *AHRR*, chromosome: 5, FDR: 0.017). In model 2, we identified two CpG sites associated with DLCO/VA; cg05575921 (Gene: *AHRR*, chromosome: 5, FDR: 0.047) and cg05951221 (Gene: chr2:233283397-233285959, chromosome: 2, FDR: 0.047).

Finally, we replicated these findings using data from the Framingham Heart Study. The statistically significant associations between DNA methylation and DLCO/VA are summarized in **Table 2**.

Table 2 Epigenome-wide associations between genome-wide DNA-methylation and DLCO/VA

Model	CpG	Chr	Pos	Gene	Discovery cohort RS			Replication cohort FHS		
					B	SE	FDR	B	SE	FDR
M1	cg05575921	5	373378	AHRR	0.065	0.012	0.017	0.037	0.0055	8.72e-06
	cg05575921	5	373378	AHRR	0.064	0.012	0.047	0.040	0.0057	1.56e-06
M2										
	cg05951221	2	233284402	-	0.049	0.0093	0.047	0.030	.0051	3.60e-04

B: effect estimate; Chr: Chromosome; DLCO/VA: Diffusing capacity of the lung for carbon monoxide by alveolar volume; FDR: false discovery rate; FHS: The Framingham Heart Study; M1: Adjusted for age, sex, white blood cell count and batch effect; M2: adjusted for age, sex, current smoking, former smoking, weight, height, white blood cell count and batch effect; Pos: position; RS: The Rotterdam Study; SE: Standard error.

Functional analysis

We used BBMRI-atlas to identify eQTMs using the identified DLCO/VA associated CpG sites (cg05575921 and cg05951221). Cg05575921 (*AHRR* gene region) was significantly associated with the expression of the *EXOC3* gene (β= 0.15 (SE=0.039), FDR= 3.90E-4). No eQTM was found for Cg05951221.

Gene Expression in lung tissue

Based on the results of the previous section, we were interested in the association between *EXOC3* and DLCO or DLCO/VA independent of smoking. Because AHRR is strongly associated with smoking, we also investigated the association between AHRR and DLCO or DLCO/VA after adjustment for smoking. Therefore, mRNA was extracted from lung resection specimens of 92 patients who underwent surgery for lung transplantation or solitary pulmonary tumours, including 44 patients without COPD

and 48 patients with COPD; and mRNA expression of *AHRR* and *EXOC3* in lung tissue was examined using quantitative RT-PCR. See **Table 3** for the general characteristics of this study population.

Table 3 Characteristics of study individuals for lung mRNA analysis (by RT-PCR) (n=92)

	Never smokers	Smokers without COPD	COPD GOLD II	COPD GOLD III-IV
Number	18	26	34	14
Gender ratio (m/f)	6/12 §	19/7 §	31/3 §	8/6 §
Age (years)	65 (56-70)	63 (55-70)	66 (58-69) ‡	56 (54-60)*† ‡
Current- / ex-smoker	NA	16/10	22/12	0/14
Pack years of smoking	NA	28 (15-45)*	45 (40-60)* ‡	30 (25-30)*† ‡

Data are presented as median (IQR)
Mann-Whitney U test: * $P < 0.05$ versus never smokers; † $P < 0.05$ versus COPD GOLD II; ‡ $P < 0.05$ versus smokers without COPD; Fisher's exact test: § $P < 0.001$

Figure 1 shows the expression levels of *AHRR* and *EXOC3* genes by smoking and COPD status. mRNA levels of *AHRR* were significantly higher in current smokers compared to never or former smokers, while mRNA levels of *EXOC3* were not significantly different between groups.

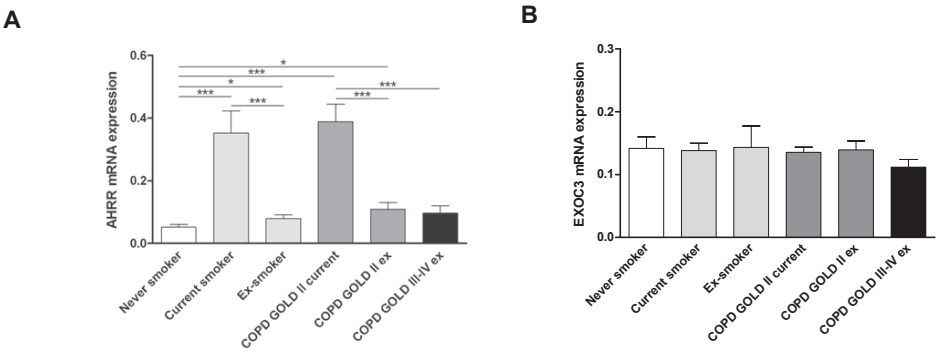

Figure 1 human lung tissue mRNA expression of the **A)** *AHRR* gene and **B)** *EXOC3* gene stratified by smoking and COPD status.
AHRR: Aryl Hydrocarbon Receptor Repressor gene; COPD: Chronic obstructive pulmonary disease; ex: ex-smoker; EXOC3: Exocyst Complex Component 3 gene. GOLD: Global initiative for chronic obstructive lung disease;

Regression analysis revealed no significant association between mRNA expression of *AHRR* and DLCO or DLCO/VA after adjustment for age, sex and smoking status in Model 1, or after additional adjustment for weight and height in Model 2.

On the other hand, mRNA expression of *EXOC3* and DLCO or DLCO/VA showed no statistically significant association in Model 1; however, the association became significant after additional adjustment for weight and height in Model 2 (see results in supplementary **Table S1**).

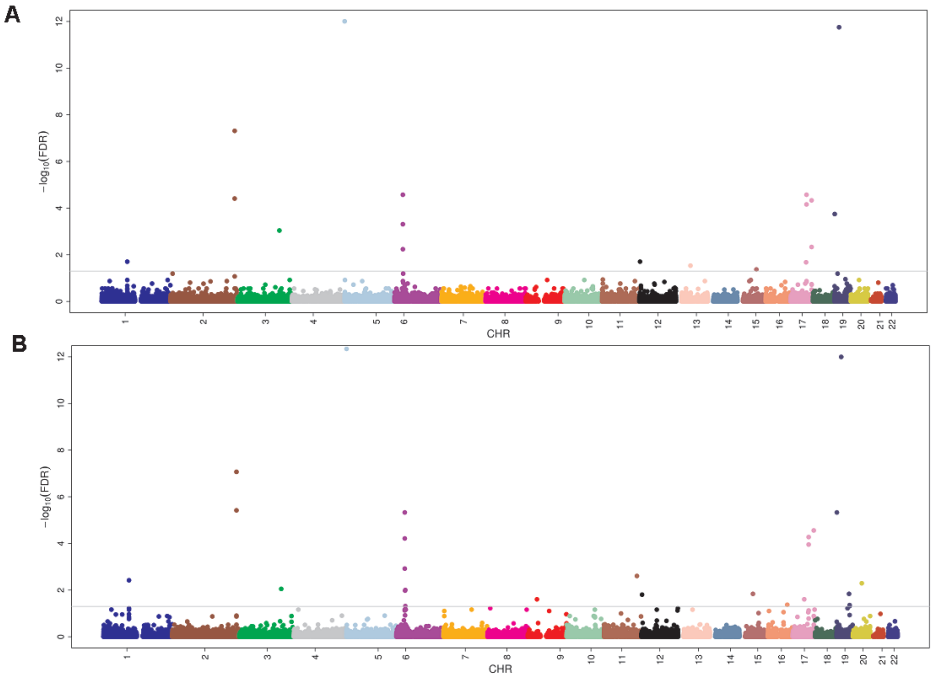

Figure 2 Manhattan plots showing the results of EWAS meta-analysis (RS and FHS) of the association between genome-wide DNA methylation and DLCO . **A)** Model1: adjusted for age, sex, smoking status, white blood cell counts and batch effects. **B)** Model2: adjusted for weight and height in addition to Model1 .

Meta-analysis

We meta-analysed the results of the analyses from the Rotterdam Study and the Framingham Heart Study for both phenotypes (DLCO and DLCO/VA) and both models. The results are summarized in the corresponding EWAS Manhattan plots in **Figures 3** and **4**, and in the supplementary tables (**Tables S1-4**). We identified 18 CpG sites which were associated with DLCO in Model 1 and 26 CpG sites in Model 2. For DLCO/VA, we identified 9 CpG sites in Model 1 and 11 CpG sites in Model 2.

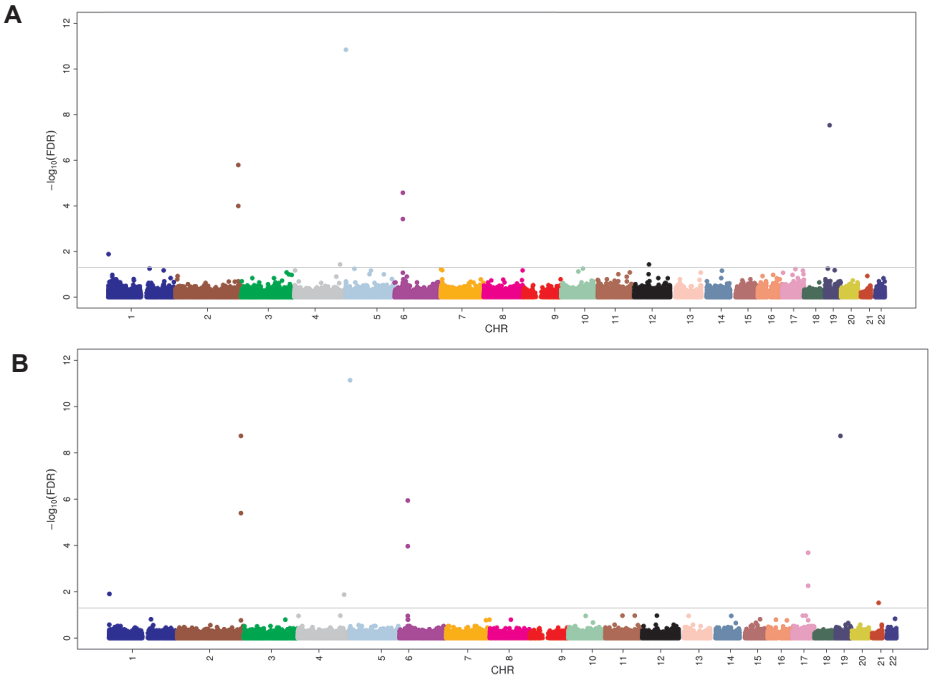

Figure 3 Manhattan plots showing the results of EWAS meta-analysis (RS and FHS) of the association between genome-wide DNA methylation and DLCO/VA. A) Model1: adjusted for age, sex, smoking status, white blood cell counts and batch effects. B) Model2: adjusted for weight and height in addition to Model1.

DISCUSSION

In this EWAS study, we investigated the epigenetic signature of DLCO and DLCO/VA as a measure of gas exchange in the lung. We observed two CpG sites with epigenome-wide associations in the Rotterdam Study, and those were replicated in the Framingham Heart Study. Additional analysis revealed a potential pathway trough which methylation of AHRR might influence gas exchange. Finally, meta-analysis of the two studies revealed many more methylation signals that need to be confirmed in future studies.

This is the first EWAS on diffusing capacity of carbon monoxide uptake in the lungs. Few studies investigated the effect of DNA methylation on lung function, and were restricted to spirometric measures (FEV_1 and FVC). Bell and colleagues (6) investigated age-related phenotypes including FEV_1 and FVC in 172 twin females. Authors reported one association at chromosome 11 in the *WT1* gene region. Another study by Marioni *et al.* (7) performed an EWAS on FEV_1 in 920 individuals and did not find epigenome-wide associations. Qui el al. performed EWAS on FEV1, FVC and COPD and found

many associations, but the analyses were not adjusted for differences in white blood cell count.

In this EWAS, we discovered and confirmed two epigenome-wide associations, one CpG site in the aryl hydrocarbon receptor repressor gene (*AHRR)* which encodes a repressor protein of the aryl hydrocarbon receptor (AHR), and the second CpG site on chromosome 2 (2q37.1). These CpG sites are well described as being strongly associated with smoking behaviour (4, 5, 15). Cg05575921 is located in the AHRR gene region and is involved in the metabolism of smoking-released chemicals, where the AHRR gene supresses the function of the AHR gene –which is responsible for the regulation of smoking related substances- through a negative feedback loop (16). Although the interaction between the genetics and DNA methylation is complex, it is believed that *AHRR* hypomethylation inhibits the translation of the gene by preventing transcription factors from binding to the promotor regions (17, 18). We hypothesise that *AHRR* hypomethylation by smoking might intervene with the elimination of the smoking related substances from the body.

In addition to the association with smoking, *AHRR* has been also recently associated with impaired lung function. Bojesen et al. (15) showed that hypomethylation of cg05575921 was associated with smoking related phenotypes such as COPD, COPD exacerbations and lung cancer. Similarly, Kodal et. al. (19) found that hypomethylation of cg05575921 was associated with low lung function, steeper lung function decline and respiratory symptoms. Whether these associations are independent of smoking effects or confounded by smoking and consequently are the results of the effect of smoking on DNA methylation, remains to be investigated.

A recent study by Li and colleagues, proved the causal effect of smoking on DNA methylation. This information eliminates the possibility for smoking to be an intermediate in the association between DNA methylation of smoking related probes and lung function. Alternatively, smoking can be a confounding factor for which we can adjust in our models. However, residual confounding is still possible as assessment of smoking status is self-reported (20). Therefore the question remains, does residual confounding by smoking explain the whole effect in the association between smoking related probes and lung function? Or is there any significant effect of DNA methylation of those smoking related probes independent of smoking? Both scenarios lead to very interesting hypotheses and might have important clinical implications. In case smoking explains the entire association, the hypothesis might be, as proposed by Li et al. (16) and Kodal et al. (19), that the association between hypomethylation of smoking related probes and a decreased lung function might reflect the smoking induced damage to the lungs through methylation of the smoking related probes. In other words, DNA methylation might act as an intermediate in the effect of smoking on smoking related diseases. The second scenario would be, that smoking affects DNA methylation, but

the effect of the hypomethylation of the smoking related probes on lung function is (partly) independent from smoking. In that case, hypomethylation of the smoking related probes might provide clinically relevant information beyond the effect of smoking on lung function. Unfortunately, it is difficult to investigate these hypotheses in cross-sectional observational studies. An alternative approach might be to study the associations between smoking related probes and lung function in never smokers. However, finding any association in never smokers is still no prove that smoking related probes are independently associated with lung function, as passive smoking or the unmeasured effects of air pollution still might bias these associations.

Regardless of the role of smoking in the association between hypomethylation of *AHRR* and a decreased pulmonary gas exchange, we propose in this study a pathway through which AHRR might affect lung function. We observed that the hypomethylation of the cg05575921 site in *AHRR* is associated with decreased expression of EXOC3 gene in whole blood. Subsequently, we were able to link a decreased expression of EXOC3 in human lung tissue to a decreased gas exchange in the lungs.

Exocyst complex component 3 (EXOC3), previously known as SEC6, is located on chromosome 5 upstream of AHRR gene and downstream of SLC9A3 gene. EXOC3 is part of the exocyst protein complex and the protein encoded by it, is involved in post Golgi trafficking and essential for biogenesis of epithelial cell surface polarity (21-23). Variation in EXOC3 is linked to variability of cystic fibrosis(24, 25). This makes EXOC3 an interesting candidate to elucidate its role in membrane pathology and gas exchange.

Strength and limitations

The strength of this study is the population-based setting with standardized data collection. Also, this study provides unique data, since we are not aware of other population-based studies with data on gas exchange. Finally, we provided additional results of gene expression analysis on lung tissue, which was performed in well-defined patient groups. However, this study also has some limitations. First, our discovery panel is relatively small, bigger sample sizes may help in the identification of more CpG sites that might give us more insight in pathology of gas exchange. Second, although we were able to replicate findings of the lead association, we cannot exclude the possibility of unmeasured confounding by smoking and air pollution. Third, this study was performed with DNA methylation data in whole blood, future studies should ideally consider performing tissue specific DNA methylation analyses. Finally, the current study analyses the data in a cross-sectional manner. As the epigenome changes over time, longitudinal data-analyses might be more informative about the epigenetic modifications over time, as it may play a crucial role in the aetiology of a decreased gas exchange.

In conclusion, impaired gas exchange is associated with smoking-related epigenetic changes. We propose a pathway through which *AHRR* hypomethylation might affect gas exchange.

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