AHRR hypomethylation, lung function, lung function decline and respiratory symptoms

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***AHRR* hypomethylation is associated with low lung function, steeper lung function decline, and respiratory symptoms** http://ow.ly/a30J30hB2dS

Cite this article as: Kodal JB, Kobylecki CJ, Vedel-Krogh S, *et al*. *AHRR* hypomethylation, lung function, lung function decline and respiratory symptoms. *Eur Respir J* 2018; 51: 1701512 [https://doi.org/10.1183/13993003.01512-2017].

ABSTRACT Epigenome-wide association studies have shown a consistent association between smoking exposure and hypomethylation in the aryl hydrocarbon receptor repressor (*AHRR*) gene (cg05575921). We tested the hypothesis that *AHRR* hypomethylation is associated with low lung function, steeper lung function decline, and respiratory symptoms in the general population.

AHRR methylation extent was measured in 9113 individuals from the 1991–1994 examination of the Copenhagen City Heart Study, using bisulfite-treated leukocyte DNA. Spirometry at the time of blood sampling was available for all individuals. Lung function was measured again for 4532 of these individuals in 2001–2003.

Cross-sectionally, a 10% lower methylation extent was associated with a 0.2 z-score (95% CI 0.1–0.2) lower forced expiratory volume in 1 s (FEV_1) after multivariable adjustment including smoking. Hypomethylation was also associated with a lower z-score for both forced vital capacity (FVC) and FEV_1/FVC. In prospective analyses, individuals in the lowest *versus* highest tertile of methylation extent had a steeper decline in $FEV_1/height^3$ (p for examination×methylation interaction=0.003) and $FVC/height^3$ (p=0.01), but not FEV_1/FVC (p=0.08). Multivariable-adjusted odds ratios per 10% lower methylation extent were 1.31 (95% CI 1.18–1.45) for chronic bronchitis and 1.21 (95% CI 1.13–1.30) for any respiratory symptoms.

AHRR hypomethylation was associated with low lung function, steeper lung function decline, and respiratory symptoms.

This article has supplementary material available from erj.ersjournals.com

Received: Jan 26 2017 | Accepted after revision: Dec 31 2017

Support statement: This study was funded by the Capital Region of Denmark, and Dept of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital. Funding information for this article has been deposited with the Crossref Funder Registry.

Conflict of interest: None declared.

Introduction

Epigenetics is the study of meiotically and mitotically heritable changes that do not entail a change in DNA sequence [1]. Evidence suggests that epigenetic mechanisms, such as DNA methylation and histone modifications, are causally involved in both monogenic and multifactorial diseases [2]. Several lifestyle factors, *e.g.* alcohol, smoking and air pollution, may influence health and disease through epigenetic mechanisms [3]. Hypomethylation in the aryl hydrocarbon receptor repressor (*AHRR*) gene has been consistently associated with smoking exposure in epigenome-wide association studies, with hypomethylation at cg05575921 showing the strongest association with smoking status [4–11]. Additionally, *AHRR* hypomethylation has been associated with second-hand smoking [12] and maternal smoking status among infants [13].

Smoking is the single most important lifestyle factor influencing lung function, lung function decline and respiratory symptoms [14, 15]. Information on smoking is often based on self-reported and inaccurate data [16]. *AHRR* hypomethylation has previously been associated with smoking [4–11, 17], spirometrically defined chronic obstructive pulmonary disease (COPD) and a higher risk of severe COPD exacerbations [17]; however, it is unknown whether *AHRR* hypomethylation is associated with low lung function, steeper lung function decline and higher risk of respiratory symptoms.

We hypothesised that *AHRR* hypomethylation, as a biomarker of smoking, offers information on lung function and respiratory symptoms beyond that offered by self-reported information on smoking. Thus, we tested the hypothesis that *AHRR* hypomethylation is associated with low lung function, steeper lung function decline and a higher risk of respiratory symptoms in the general population.

Methods

Study population

The Copenhagen City Heart Study (CCHS) is a prospective cohort study of the Danish general population initiated in 1976–1978 with follow-up examinations in 1981–1983, 1991–1994 and 2001–2003 [17, 18]. Individuals aged 20–100 years were selected randomly from the Danish Central Person Registry to represent the Danish general population. At each examination participants filled out an extensive questionnaire reflecting lifestyle and health. The questionnaire was reviewed by the participant together with an investigator on the day of study attendance, prior to a physical examination and blood sampling for biochemical analysis and DNA extraction. In the present study, we included 9113 individuals from the 1991–1994 examination (61% of all invited) with methylation extent measurements and spirometry available. Additionally, all living participants from the 1991–1994 examination were re-invited for the examination in 2001–2003, and 4581 (50%) participated, thus allowing repeated measurements of lung function for a subset of individuals. The study was approved by Herlev and Gentofte Hospital and a Danish ethics committee (KF100.2039/91), and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Methylation extent

DNA was extracted from frozen whole blood samples from the 1991–1994 examination using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The extent of methylation of *AHRR* (corresponding to the cg05575921 CpG site on the Illumina BeadChip 450 K array) was measured in duplicate samples using bisulfite treatment followed by PCR using a Taqman-based assay with probes designed to detect either the unmethylated and therefore conversed T residue, or the methylated and therefore conserved C residue, as previously described [17]. Failed measurements were repeated and valid measurements of methylation extent were available for >99.8% of available DNA samples. At the level of 71% methylation extent, coefficients of variation varied from 5% to 7% for different lots of the internal control [17]. Measurements were adjusted for 13 batches to account for inter-assay variation, and validated using pyrosequencing [17].

Spirometry

Forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were determined using a dry wedge spirometer at the 1991–1994 and 2001–2003 examinations. For each individual, spirometry was performed in triplicate, and results were only accepted if the variation between the two best-performing of these was <5%.

Covariates

Information on smoking status, cumulative smoking, exposure to passive smoking, exposure to occupational dust and fumes, and highest completed education level were self-reported. Body mass index (BMI) was calculated as measured weight in kilograms divided by measured height in metres squared. We did not have information on traffic air pollution exposure.

Respiratory symptoms

All included respiratory symptoms were self-reported from the 1991–1994 examination. Additional details on methods are provided in the supplementary material.

Statistical analysis

We used Stata/SE 13.1. A two-tailed p-value <0.05 was considered significant. We used Cuzick's nonparametric test for trend to test for associations of *AHRR* methylation extent for never-smokers, former smokers and current smokers. Kernel density plots stratified by smoking status were used to visualise the distribution of methylation extent. Additionally, methylation extent in former smokers by time since smoking cessation was plotted for the whole population as well as stratified by sex. The associations of methylation extent with sex, height and age were assessed in linear models stratified by smoking status. Missing data on covariates (0.2%) were imputed using multivariable chained imputations according to age and sex; however, without imputation results were similar.

Lung function: cross-sectional analyses

Linear regression models were used in cross-sectional analyses of the association between lung function z-scores and methylation extent. FEV_1, FVC and FEV_1/FVC z-scores were calculated according to the Global Lung Function Initiative 2012 equations (GLI-2012) [19]. We assessed homoscedasticity visually using a plot of residuals *versus* predicted values. We assessed the normality of residuals by plotting quantiles of the variable against quantiles of the normal distribution. No major violations of homoscedasticity or normality were observed. A quadratic regression line was plotted as well as a linear regression line for visual assessment. To formally test for non-linearity, a quadratic methylation extent term was introduced and a likelihood ratio test was performed to test for similarity of coefficients between the linear and quadratic models. We performed multivariable adjustments for relevant confounders, *i.e.* sex, age (continuous), BMI (quintiles with second quintile as the reference), passive smoking (dichotomous), dust and fumes exposure (dichotomous), educational level (in conventional Danish categories: <10 years of education, 10 years of education, higher education <1 year, higher education 1–3 years, higher education >3 years, and academic degree), smoking status (current, former or never-smokers) and cumulative smoking measured in pack-years (continuous). Furthermore, analyses were stratified by both sex and smoking status.

Lung function decline: prospective analyses

The following measures were used to assess lung function: FEV_1/baseline height cubed (height^3) (previously used by Fletcher and Peto [20]), FVC/height^3 and FEV_1/FVC. The decline in these measures was assessed using repeated measures linear mixed models, because these models can account for within-subject correlation [21]. The number of examinations (one or two) specified the number of repeated measurements of lung function; those individuals with only one measurement were included to increase precision of the baseline estimate. For the purpose of graphing the association, tertiles of methylation extent were used. We used an unstructured covariance type because it places no restriction on structure. The identity of each individual was introduced as a random effect to specify the grouping structure, thereby accounting for within-subject correlation. We tested for an interaction between methylation extent and time from first to second examination on change in lung function using a likelihood ratio test by introducing a two-factor interaction term (examination number and methylation extent in tertiles) in a model that included both factors. Furthermore, we stratified lung function changes by smoking status and sex. Models were adjusted for those variables, *i.e.* age, sex, examination (1991–1994 or 2001–2003) and cumulative smoking (updated at each examination), which could confound the associations among methylation extent and lung function parameters. Examples of group averages were calculated using linear combinations of coefficients from the mixed model linear regression.

In a sensitivity analysis, we included only individuals who attended both examinations in a linear regression model. To assess potential bias from exclusion of individuals who only attended the first examination, we assessed differences in the occurrence of COPD hospitalisations, death, respiratory symptoms and spirometry between individuals who attended one or two examinations.

Methylation extent and the difference between measured FEV_1 and predicted FEV_1

The association between methylation extent and the difference between measured FEV_1 and predicted FEV_1 was assessed using an unadjusted linear regression model. For all individuals, we plotted measured FEV_1 minus predicted FEV_1 against methylation extent in the 1991–1994 examination. For individuals attending both examinations, we also plotted measured FEV_1 minus predicted FEV_1 in the 2001–2003 examination against methylation extent in the 1991–1994 examination.

Respiratory symptoms: cross-sectional analyses

The association between respiratory symptoms and methylation extent stratified by smoking status was assessed by a cross-sectional design using logistic regression. Multivariable models were adjusted for the same confounders as in the linear regression models including FEV_1 z-score.

In sensitivity analyses, we performed logistic regression between methylation extent and respiratory symptoms stratified by both sex and smoking status.

Results

Table 1 summarises baseline characteristics at the 1991–1994 examination according to smoking status and tertiles of methylation extent. The median methylation extent in the 9113 individuals from the CCHS was 56% (interquartile range (IQR) 50–63). As shown previously [17], the methylation extent of *AHRR* differed with smoking status (figure 1a); for current smokers, the median methylation extent was 50% (IQR 47–54), for former smokers 59% (IQR 54–64) and for never-smokers 64% (IQR 60–68) (p for trend <0.001). When stratified by sex, differences in methylation extent were most pronounced in men (supplementary figure S1). We found that the longer the smoking abstinence, the higher the methylation extent of *AHRR* (figure 1b). When stratified by smoking status, the methylation extent was consistently associated with sex in all strata, with a lower methylation extent in men than in women (supplementary table S1). In both sexes, methylation extent was positively associated with smoking abstinence time (both p-values for trend <0.001, supplementary figure S1).

Lung function: cross-sectional analyses

In a multivariable adjusted analysis, low methylation extent was associated with low FEV_1 z-score (figure 2). A 10% lower methylation extent was associated with a 0.2 z-score (95% CI 0.1–0.2) lower FEV_1 (figure 2a). Similar results were seen for z-scores of FVC (figure 2b) and FEV_1/FVC (figure 2c). Results were similar when stratified by smoking status (supplementary figure S2), although somewhat attenuated in never-smokers. Adding a quadratic term to the regression did improve the fit of the model for some parameters; however, most deviations were observed at the highest and lowest methylation levels (figure 2 and supplementary figure S2). Likewise, results were similar in women and men separately (supplementary figures S3 and S4).

Lung function decline: prospective analyses

In prospective analyses of change in lung function over time, we found that the examination (1991–1994 *versus* 2001–2003) interacted with tertiles of methylation extent on the decline of $FEV_1/height^3$ (p for

TABLE 1 Differences in baseline characteristics of individuals according to tertiles of aryl hydrocarbon receptor repressor (*AHRR*) methylation extent and smoking status

Characteristic	All	Methylation extent tertiles			Smoking status		
		Lowest	Middle	Highest	Current smokers	Former smokers	Never-smokers
Individuals n	9113	3058	3053	3002	4426	2356	2331
Methylation extent %	56 (50–63)	48 (46–50)	56 (54–58)	66 (63–69)	50 (47–54)	59 (54–64)	64 (60–68)
Males	4065 (45)	1580 (52)	1445 (47)	1040 (35)	2112 (48)	1209 (51)	744 (32)
Age years	60 (47–70)	58 (47–67)	61 (48–71)	60 (45–71)	58 (47–67)	64 (52–72)	57 (42–71)
FEV_1 z-score	−0.6 (−1.4–0.3)	−0.9 (−1.8–0.1)	−0.6 (−1.4–0.2)	−0.2 (−0.9–0.6)	−0.8 (−1.7–0)	−0.4 (−1.3–0.3)	−0.2 (−0.9–0.6)
FEV_1/FVC	0.79 (0.73–0.83)	0.76 (0.70–0.81)	0.78 (0.73–0.83)	0.81 (0.76–0.85)	0.77 (0.70–0.82)	0.79 (0.73–0.83)	0.81 (0.77–0.85)
Exposed to passive smoking	3283 (36)	1453 (48)	1029 (34)	801 (27)	2109 (48)	615 (26)	559 (24)
Occupational exposures to dust and fumes	1689 (19)	768 (25)	546 (18)	375 (12)	980 (22)	443 (19)	266 (11)
Completed higher education	1837 (20)	435 (14)	641 (21)	761 (25)	707 (16)	568 (24)	562 (24)
Body mass index $kg \cdot m^{-2}$	25 (22–28)	24 (22–27)	25 (23–28)	25 (23–28)	24 (22–27)	26 (23–29)	25 (22–28)
Never-smokers	2331 (26)	34 (1.1)	607 (20)	1690 (56)			
Current smokers	4426 (49)	2705 (88)	1391 (46)	330 (11)			
Former smokers	2356 (26)	319 (10)	1055 (35)	982 (33)			
Cumulative smoking pack-years[#]	26 (14–40)	32 (21–45)	25 (13–40)	13 (5.0–25)	30 (18–43)	20 (8.5–35)	0

Data are presented as n (%) for categorical values and median (IQR) for continuous values, unless otherwise stated. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; IQR: interquartile range. [#]: Calculated for current and former smokers only.

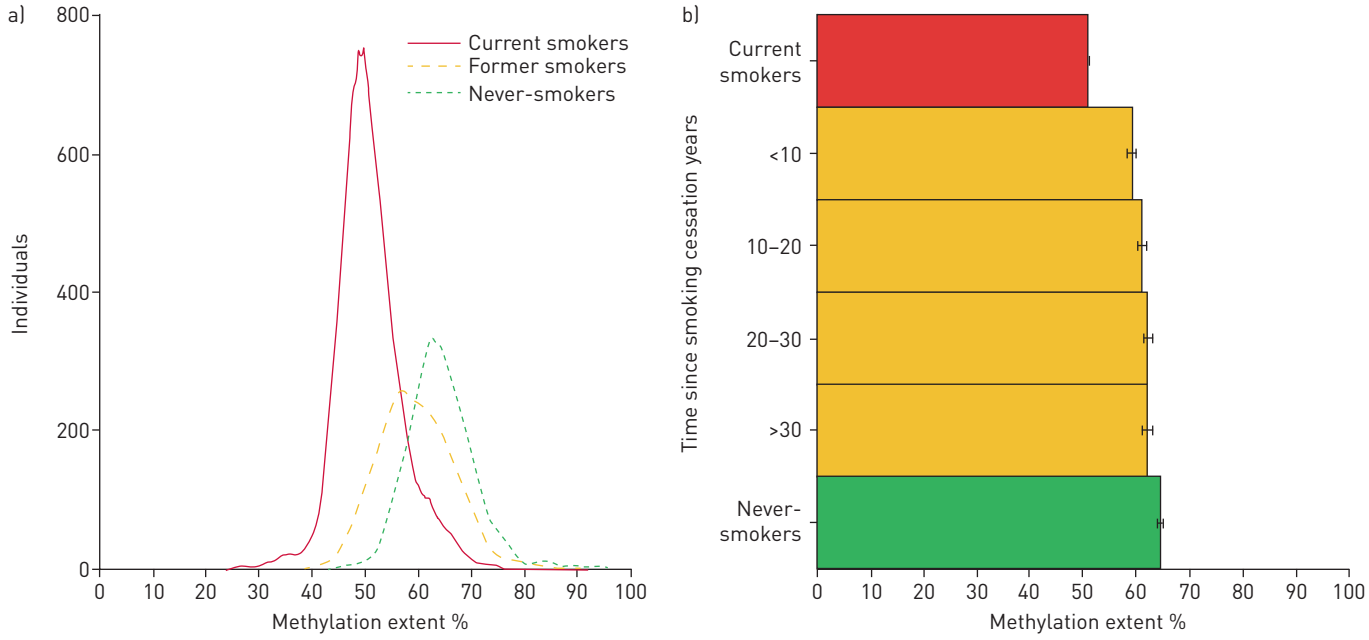

FIGURE 1 Distribution of aryl hydrocarbon receptor repressor (*AHRR*) methylation extent in 9113 individuals. a) Distribution of methylation extent among current smokers, former smokers and never-smokers. b) The mean values of methylation extent with 95% confidence intervals for subgroups of former smokers according to years since smoking cessation. Current and never-smokers are shown for reference. The black bars represent the 95% confidence interval. In former smokers, p for trend was calculated using Cuzick's test for trend: p<0.001.

interaction=0.003) (figure 3). Individuals in the lowest methylation extent tertile had a steeper decline in $FEV_1/height^3$ compared to individuals in the highest methylation extent tertile. In the lowest methylation extent tertile, based on longitudinal models, male participants (at age 50, height 1.80 m, 5 pack-years smoked) had an average FEV_1 of 3.71 L (95% CI 3.68–3.75) in the 1991–1994 examination and 3.16 L (95% CI 3.12–3.20) in the 2001–2003 examination. The corresponding estimates for individuals in the highest methylation extent tertile were 3.97 L (95% CI 3.94–4.00) and 3.44 L (95% CI 3.41–3.47), respectively (data not shown). When stratified by sex, the steeper decline of $FEV_1/height^3$ in individuals with low methylation extent was most pronounced in men (supplementary figure S5). When stratified by smoking status, results were similar in current smokers, whereas there was no association between decline in $FEV_1/height^3$ and *AHRR* hypomethylation among never-smokers (figure 3). Similar results with a steeper decline in individuals with the lowest *versus* highest tertile of methylation extent were seen for $FVC/height^3$ (p for interaction <0.001) and for FEV_1/FVC (p for interaction <0.001) (supplementary figures S6 and S7). After additional adjustment for pack-years updated at both examinations, no interaction of examination with methylation extent tertile was found for FEV_1/FVC (p for interaction=0.08). Similar trends were seen for both FVC and FEV_1/FVC for women and men in stratified analyses (supplementary figures S8 and S9). In a sensitivity analysis including only individuals with two measurements, we found a decline in FEV_1 of 3.6 mL/year (95% CI 2.2–5.0) associated with a 10% lower methylation extent (supplementary figure S10). However, among individuals attending only the 1991–1994 examination, there was a higher occurrence of death within 15 years, more COPD-related hospitalisations and more reported respiratory symptoms than among individuals attending both examinations (supplementary table S2).

Methylation extent and the difference between measured FEV_1 and predicted FEV_1

The difference between measured FEV_1 and the predicted FEV_1 by GLI-2012 equations was associated with methylation extent both cross-sectionally (figure 4a) and prospectively (figure 4b). The difference between measured FEV_1 and predicted FEV_1 was 0.16 L (95% CI 0.15–0.18) higher per 10% lower methylation extent in the 1991–1994 examination. Prospectively, methylation extent was associated with a 0.15 L (95% CI 0.13–0.17) higher difference per 10% lower methylation extent in the 2001–2003 examination.

Respiratory symptoms: cross-sectional analyses

In stratified analyses, after multivariable adjustments including FEV_1 z-score, methylation extent was associated with all of the respiratory symptom categories in current smokers, but only chronic bronchitis,

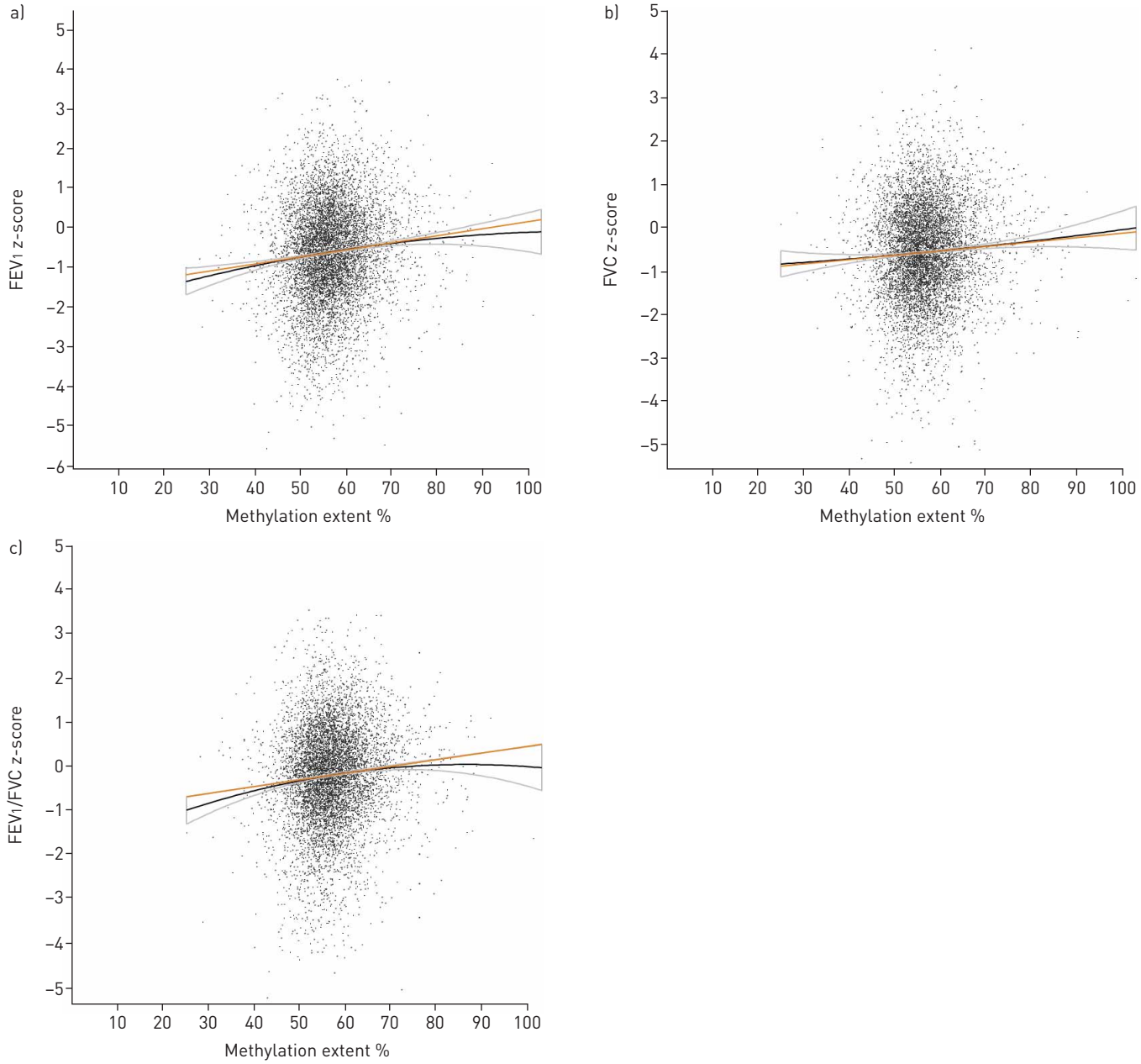

FIGURE 2 Associations of aryl hydrocarbon receptor repressor (*AHRR*) methylation extent with lung function parameters. a) FEV_1 is 0.2 z-score (95% CI 0.1–0.2) lower per 10% lower methylation extent; b) FVC is 0.1 z-score (95% CI 0.07–0.1) lower per 10% lower methylation extent; and c) FEV_1/FVC is 0.2 z-score (95% CI 0.1–0.2) lower per 10% lower methylation extent. Analyses were multivariable adjusted for age, sex, body mass index, dust and fume exposure, passive smoking, educational level, smoking status, and cumulative smoking. The orange line represents a linear regression line. The black line represents a quadratic regression line. The area surrounding the quadratic line represents the 95% confidence interval of the regression line. The black dots represent individual measurements. The individual measurements are adjusted for covariates. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.

wheezing and any respiratory symptoms were significant in former smokers (figure 5). Only chronic bronchitis was significantly associated with methylation extent in all smoking strata. In all individuals, multivariable adjusted analyses that included smoking status and cumulative smoking showed that methylation extent was associated with all respiratory symptoms except dyspnoea (supplementary figure S11). For chronic bronchitis, which displayed the strongest association with methylation extent, the multivariable adjusted odds ratio per 10% lower methylation extent was 1.31 (95% CI 1.18–1.45) and 1.21 (95% CI 1.13–1.30) for any respiratory symptoms.

After further stratification by sex, results were similar in men and women separately although the associations among former smokers were most pronounced in men (supplementary figure S12).

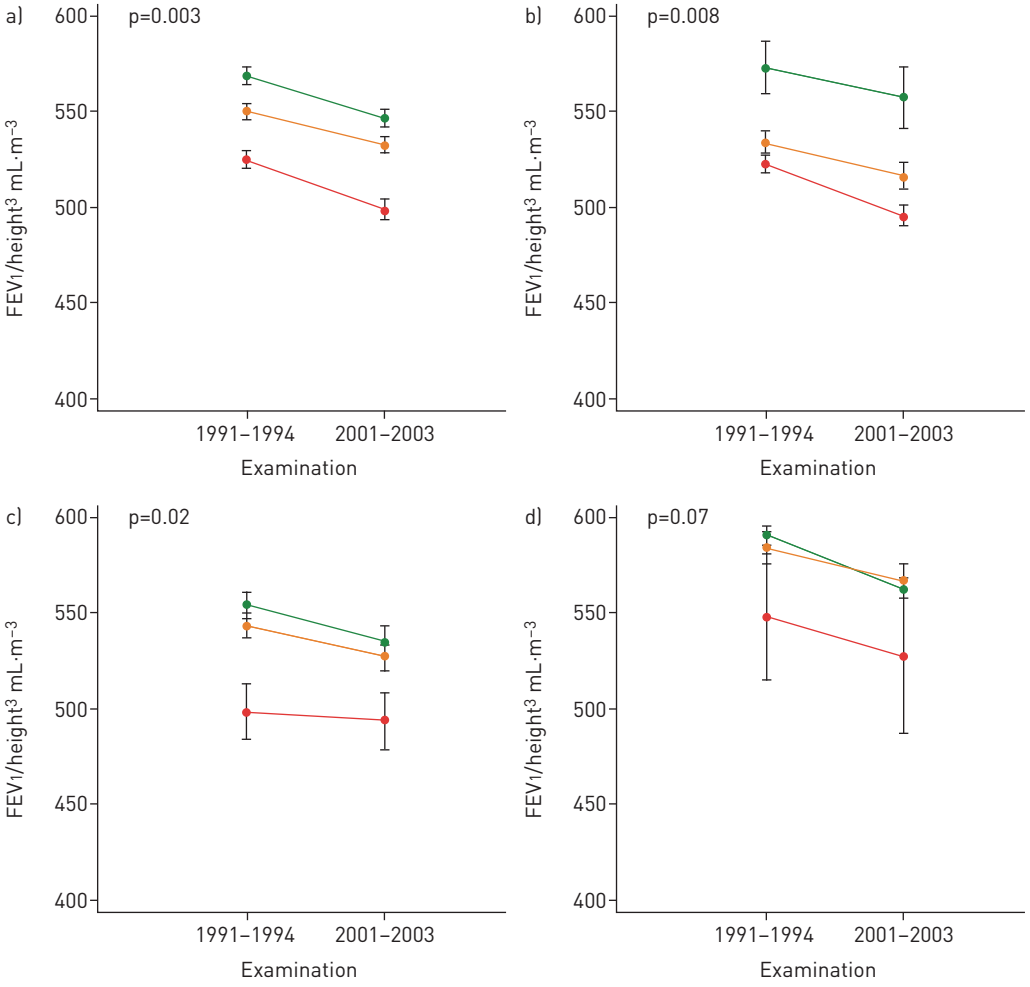

FIGURE 3 Relationship between changes in $FEV_1/height^3$ according to tertiles of aryl hydrocarbon receptor repressor (*AHRR*) methylation a) overall and stratified into b) current smokers, c) former smokers and d) never-smokers. Analyses were adjusted for cumulative smoking updated at each examination, age, sex and examination session. Data based on one to two spirometries for each of 9113 individuals, spanning up to 11.5 years; those individuals with only one measurement were included to increase precision of the baseline estimate. The identity of each individual was introduced as a random effect to specify the grouping structure, thereby accounting for within-subject correlation. Only baseline height was used. The black bars represent the 95% confidence interval. The green line represents the highest methylation extent tertile; the orange line represents the middle methylation extent tertile; the red line represents the lowest methylation extent tertile. p-values for interaction of examination (1991–1994 *versus* 2001–2003) with tertiles of methylation extent are reported. FEV_1: forced expiratory volume in 1 s.

Discussion

In this study of 9113 individuals from the general population, we found that *AHRR* hypomethylation was associated with low lung function, steeper lung function decline and a higher risk of respiratory symptoms. These are novel findings.

Although a role for AHRR in lung function impairment is currently unclear, these findings have several implications. First, they suggest that methylation extent measurements may offer information on smoking not captured by self-reported smoking behaviour. Second, because hypomethylation was also associated with low lung function and increased risk of chronic bronchitis among never-smokers, hypomethylation may capture unreported tobacco exposure. Third, hypomethylation may serve as a marker of susceptibility to the harmful consequences of smoking and help identify smokers more prone to tobacco-induced lung damage. Fourth, although a study like ours cannot infer causality, *AHRR* hypomethylation may be on the causal pathway between smoking and lung function impairment, lung function decline and respiratory symptoms. These considerations are based on the sole assumption of an effect of smoking on *AHRR* methylation; however, the results in never-smokers and the fact that cigarette smoke contains many toxicants means that we cannot rule out the effect of other environmental exposures, *e.g.* air pollution.

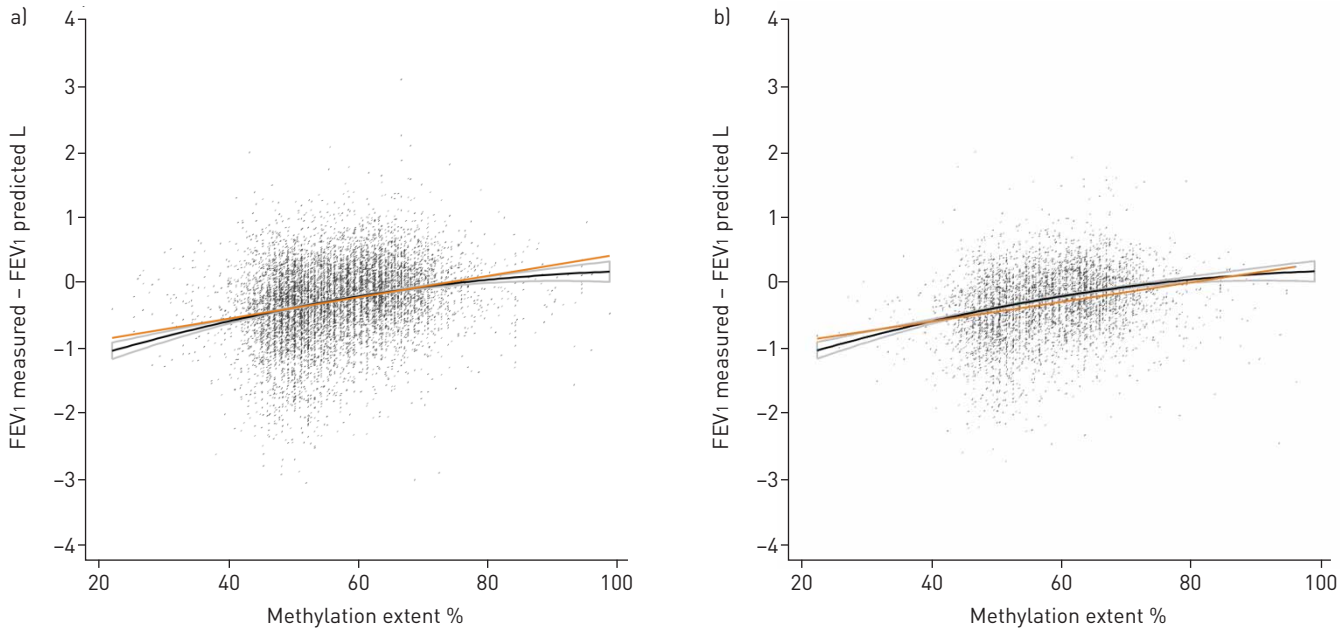

FIGURE 4 Aryl hydrocarbon receptor repressor (*AHRR*) methylation extent and the difference between measured FEV_1 and predicted FEV_1 a) cross-sectionally and b) prospectively. In a, the methylation extent measured in 1991–1994 is plotted against measured FEV_1 and predicted FEV_1 in the 1991–1994 examination; FEV_1 measured – FEV_1 predicted=0.16 L (95% CI 0.15–0.18) lower per 10% lower methylation extent. In b, the methylation extent measured in 1991–1994 is plotted against measured FEV_1 and predicted FEV_1 in the 2001–2003 examination; FEV_1 measured – FEV_1 predicted=0.15 L (95% CI 0.13–0.17) lower per 10% lower methylation extent. Predicted FEV_1 was calculated using the global lung function 2012 equations based on age, height, sex and ethnicity. The orange line represents an unadjusted linear regression line. The black line represents an unadjusted quadratic regression line. The area surrounding the quadratic regression line represents the 95% confidence interval of the regression line. The black dots represent individual measurements. FEV_1: forced expiratory volume in 1 s.

AHRR hypomethylation is a potential biomarker of smoking history and captures former smoking even after >35 years of smoking cessation [22]. Self-reported smoking status and cumulative smoking are often insufficiently reported and the latter may be subject to recall bias [16, 23]. Thus, more objective markers of smoking behaviour are needed. Currently, cotinine concentrations in blood or urine, or measurements of exhaled CO concentrations, both with biological half-lives <24 h, are the main biomarkers to validate smoking status [10]. While these may be able to distinguish true smokers from non-smokers they are not well suited for reflecting long-term smoking history [10, 22]. The concept of *AHRR* methylation extent as

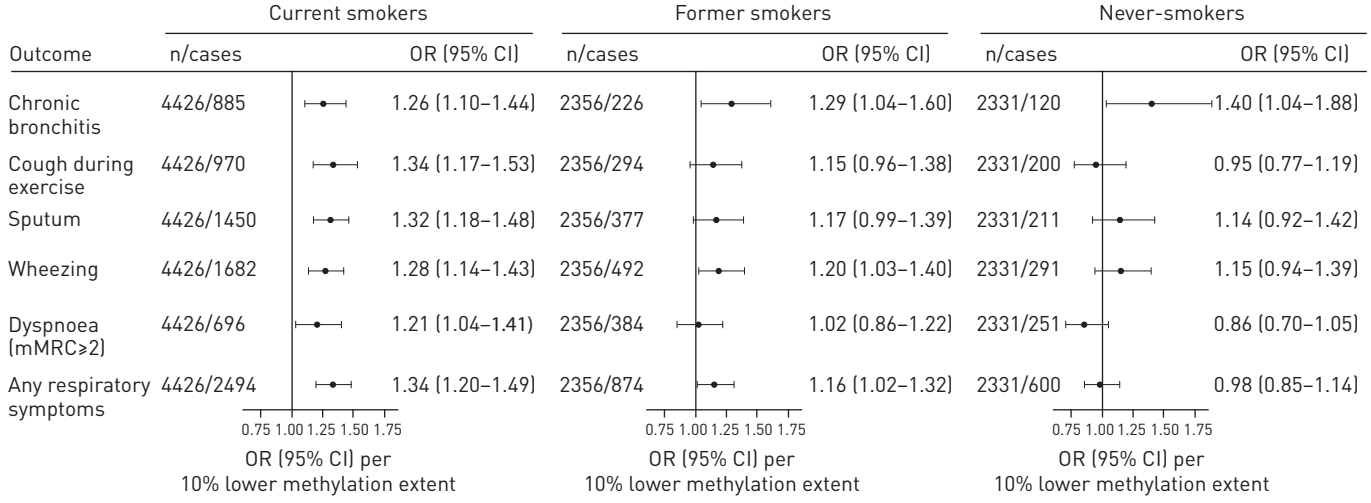

FIGURE 5 Odds ratio for respiratory symptoms per 10% lower aryl hydrocarbon receptor repressor (*AHRR*) methylation extent stratified by smoking status at the 1991–1994 examination. All analyses are adjusted for age, sex, body mass index, dust and fume exposure, passive smoking, educational level, forced expiratory volume in 1 s z-score and cumulative smoking (former and current smokers only). mMRC: modified Medical Research Council dyspnoea scale.

a biomarker of long-term smoking history [17] is supported by our findings that *AHRR* methylation extent in former smokers is associated with duration of abstinence as well as with respiratory symptoms. However, whether these associations can be ascribed to hypomethylation being a better indicator of the damaging effects of smoking or to residual confounding due to inadequate reporting of smoking habits remains to be clarified. Likewise, the association between *AHRR* hypomethylation and low lung function and chronic bronchitis in never-smokers may be due to residual confounding, or reflect other non-measured environmental exposures, such as air pollution. Hypomethylation was associated with a larger difference between measured FEV_1 and predicted FEV_1 both cross-sectionally and prospectively 10 years later. We speculate that CpG sites such as *AHRR* could account for part of this difference and hence improve prediction models for lung function and lung function decline.

Alternatively, *AHRR* hypomethylation may be a proxy for susceptibility to tobacco-induced lung damage. Mechanistically, double-stranded DNA breaks caused by tobacco smoke constituents lead to DNA repair and recruitment of DNA methyltransferases [24]. *De novo* methylation of the CpG dinucleotides adjacent to the repaired DNA may occur to avoid the expression of mutant protein through gene silencing [24]. Smoking has the ability to alter gene methylation through multiple pathways, one being through nicotine-induced downregulation of DNA methyltransferase I expression [24–26]. Thus, if *AHRR* hypomethylation marks DNA damage and insufficient repair mechanisms, it may identify individuals with more DNA damage from tobacco exposure relative to tobacco-exposed individuals with normal methylation levels.

Finally, *AHRR* hypomethylation may be on the causal pathway of smoking-induced lung damage. Given the many mechanisms by which smoking causes lung injury, it is unclear which constituent of cigarette smoke is monitored by *AHRR* hypomethylation. Our observational study cannot clarify this important point. *AHRR* hypomethylation is associated with higher AHRR expression in monocytes [27], lymphoblasts and pulmonary macrophages in smokers [28]. Although the complex interplay of AHRR in the AHR pathway is still under investigation, studies suggest that increased AHRR expression represses AHR activity through negative feedback [29], entailing decreased expression of xenobiotic metabolising genes such as *CYP1A1* [25]. In turn, this may compromise the body's ability to metabolise and remove harmful agents such as polyaromatic hydrocarbons, potentially leading to impaired lung function [30]. Alternatively, given that *AHRR*-deficient mice show lower expression of certain proinflammatory molecules after lipopolysaccharide injection [31], AHRR may be involved in regulation of the inflammatory response that is also present in declining lung function. However, we cannot exclude the possibility that methylation changes for intragenic methylation such as *AHRR* cg05575921 may be a consequence of changes in gene expression [32]. Thus, *AHRR* hypomethylation may be secondary to smoke-induced *AHRR* expression and therefore not a causal factor alone.

In our study we found several sex differences. First, in all smoking strata, men had a lower methylation extent than women in models adjusted for age and height. This may be due to differences in smoking behaviour and general environmental exposures between men and women, or could reflect a higher susceptibility to tobacco-induced DNA alterations in men. Second, in women, the methylation extent did not discriminate well between former and never-smokers and was not associated with respiratory symptoms among former smokers. Last, in prospective analyses of women, low methylation extent was not associated with a steeper lung function decline in any smoking strata. One explanation for this could be the differences in reporting of smoking behaviour between men and women, or again these findings may reflect actual biological differences which could be addressed in future studies.

Previous studies on *AHRR* hypomethylation and respiratory diseases have mainly focused on lung cancer; *AHRR* hypomethylation is associated with lung cancer incidence, lung cancer mortality and all-cause mortality [5, 7, 17]. Furthermore, data from the CCHS has shown an association between *AHRR* hypomethylation and spirometrically defined COPD in cross-sectional analyses, as well as a higher risk of future severe COPD exacerbations [17]. Now, we show that *AHRR* hypomethylation is associated with low lung function, steeper lung function decline and a higher risk of respiratory symptoms.

The strengths of our study include the large sample size from a homogenous population and repeated measurements of lung function. Still, some potential limitations should be considered. First, bias may have been introduced by the use of repeated spirometry; because *AHRR* hypomethylation is associated with lung function and all-cause mortality [17], individuals with low methylation extent may not have attended both examinations owing to morbidity or death, as shown in our study. However, this preferential removal of individuals with low methylation extent would most likely bias our results towards the null hypothesis and thus cannot explain our findings. Second, because our study population consisted solely of Danes, our findings may not necessarily be applicable to individuals of different ethnicities, although, for now, no evidence exists to support this.

In conclusion, *AHRR* hypomethylation among individuals from the general population was associated with low lung function, steeper lung function decline and a higher risk of respiratory symptoms. Our results extend the number of smoking-related phenotypes associated with *AHRR* hypomethylation and strengthen the evidence for *AHRR* hypomethylation as a potential biomarker of smoking history and/or harmful effects thereof. The role and complex interplay of AHRR and AHR in lung function impairment, lung function decline and the development of respiratory symptoms remains to be clarified.

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