ERJ Express. Published on January 18, 2018 as doi: 10.1183/13993003.01512-2017

EUROPEAN RESPIRATORY *journal*

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Original article

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

Jakob B. Kodal, Camilla J. Kobylecki, Signe Vedel-Krogh, Børge G. Nordestgaard, Stig E. Bojesen

Please 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; in press (https://doi.org/10.1183/13993003.01512-2017).

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

Jakob B. Kodal[1,2], Camilla J. Kobylecki[1,2], Signe Vedel-Krogh[1,2], Børge G. Nordestgaard[1,2,3], Stig E. Bojesen[1,2,3]*

[1]Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital, Denmark

[2]Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

[3]The Copenhagen City Heart Study, Frederiksberg Hospital, Copenhagen University Hospital, Denmark

Financial support: The Capital Region of Denmark, Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital.

***Corresponding author:**

Stig E. Bojesen, M.D., PhD, D.M.Sc.

Department of Clinical Biochemistry

Herlev and Gentofte Hospital, Copenhagen University Hospital

Herlev Ringvej 75, DK-2730 Herlev, Denmark

E-mail: stig.egil.bojesen@regionh.dk, Phone: +45 3868 3843, Fax: +45 3868 3311

“Take home” message: *AHRR* hypomethylation is associated with low lung function, steeper lung function decline, and respiratory symptoms.

Abstract

Introduction: 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.

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

Results: Cross-sectionally, a 10% lower methylation extent was associated with a 0.2 z-score(95% confidence interval:0.1-0.2) lower forced expiratory volume in 1 second(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(1.18-1.45) for chronic bronchitis and 1.21(1.13-1.30) for any respiratory symptoms.

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

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], and several lifestyle factors, such as 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 both second hand smoking[12] and with maternal smoking status among infants[13].

The single most important lifestyle factor influencing lung function, lung function decline, and respiratory symptoms is smoking[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 hypothesized that *AHRR* hypomethylation, as a biomarker of smoking, offers additional information with respect to 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 with 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 life-style and health. The questionnaire was reviewed by the participant together with an investigator at the day of study attendance, prior to 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, 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 Qiagen Blood Kits. *AHRR* (corresponding to the cg05575921 CpG site on the Illumina BeadChip 450K array) methylation extent was measured in duplicate samples using first 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 samples were remeasured and valid measurements of methylation extent were available for more than 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 second (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 variation between the two best-performing of these was less than 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 meters 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 online supplement.

Statistical analysis

We used Stata/SE 13.1. 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 through never, former, and current smokers. In order to visualize the distribution of methylation extent, kernel density plots stratified by smoking status were graphed. Additionally, methylation extent in former smokers by time since smoking cessation were plotted for the whole population as well as stratified by sex. The associations between methylation extent and 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, and for normality of residuals by plotting quantiles of the variable against quantiles of the normal distribution. No major violations of homoscedasticity or normality were observed. Additionally, 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, that is, 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 year of education, 10 years of education, higher education <1 year, higher education of 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 smoking status and by sex.

Lung function decline: prospective analyses

Decline in FEV_1/baseline height cubed ($height^3$) previously used by Fletcher and Peto[20], $FVC/height^3$, and FEV_1/FVC was assessed using repeated measures linear mixed models, as these models can account for within-subject correlation[21]. The number of examinations (1 or 2) 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 was used. An unstructured covariance type was used as it places no restriction on structure. Identity of each individual was introduced as a random effect to specify the grouping structure, hereby accounting for within-subject correlation. We tested for 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 also including both factors. Furthermore, we stratified

lung function changes by smoking status and sex. Models were adjusted for variables which could confound the association among methylation extent and decline in lung function parameters, that is, age, sex, examination (1991-1994 or 2001-2003), and cumulative smoking, which were updated at each examination. 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. In order to assess potential bias from exclusion of individuals who only attended the first examination, we assessed differences in occurrence of COPD hospitalizations, 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 measured FEV_1 minus 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, the methylation extent at the first examination and the association with measured FEV_1 minus predicted FEV_1 in the 2001-2003 examination were also plotted.

Respiratory symptoms: cross-sectional analyses

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

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

Results

Table 1 summarizes baseline characteristics at the 1991-1994 examination according to smoking status and tertiles of methylation extent. In the 9113 individuals from the CCHS, the median methylation extent was 56% (interquartile range (IQR): 50-63). As shown previously[17] methylation extent of *AHRR* differed with smoking status (Figure 1 upper panel); for current smokers, the median methylation extent was 50% (IQR: 47-54), for former smokers 59% (54-64), and for never smokers 64% (60-68) (p for trend <0.001). Stratified by sex, differences in methylation extent were most pronounced in men (Supplementary Figure S1). Furthermore, we found that the longer the smoking abstinence, the higher the methylation extent of *AHRR* (Figure 1 lower panel).When stratified on smoking status, 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. Similar results were seen for z-scores of FVC and FEV_1/FVC. Results were similar when stratified on 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 an interaction of examination (1991-1994 versus 2001-2003) with tertiles of methylation extent on the decline of $FEV_1/height^3$ (p for interaction=0.003)(Figure 3). Individuals in the lowest tertile of methylation extent had a steeper decline in $FEV_1/height^3$ compared to individuals in the highest tertile of methylation extent. In the lowest tertile of methylation extent, 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 liter (95%CI: 3.68-3.75) in the 1991-1994 examination and 3.16 liter (3.12-3.20) in 2001-2003 examination. The corresponding estimates for individuals in the highest tertile of methylation extent were 3.97 (3.94-4.00) and 3.44 (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 on smoking status, results were similar in current smokers, while there was no association between decline in $FEV_1/height^3$ and *AHRR* hypomethylation among never smokers (Figure 3). Similar results with 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 Figure S6 and S7). After additional adjustment for pack-years updated at both examinations, no interaction of examination with tertiles of methylation extent was found for FEV_1/FVC (p for interaction 0.08). Similar trends were seen for both FVC and FEV_1/FVC for both women and men in stratified analyses (Supplementary Figure S8 and S9). In a sensitivity analysis including only individuals with two measurements, we found a FEV_1 decline of 3.6ml/year (95%CI:2.2-5.0) associated with a 10% lower methylation extent (Supplementary Figure S10). However, individuals only attending the 1991-1994 examination had higher occurrence of death within 15 years, more COPD related hospitalizations, and reported more respiratory symptoms than 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 4, upper panel) and prospectively (Figure 4, lower panel). The difference between FEV_1 measured and FEV_1 predicted 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 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, 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 including smoking status and cumulative smoking was associated with all respiratory symptoms except dyspnea (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 (1.18-1.45) and 1.21 (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).

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 with higher risk of respiratory symptoms. These are novel findings.

Although a role for AHRR in lung function impairment is currently unclear, these findings may have several implications. First, they suggest that methylation extent measurements may offer information on smoking not captured by self-reported smoking behavior. Second, as hypomethylation was also associated with low lung function and 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 many toxicants are present in cigarette smoke, should not rule out the effect of other environmental exposures, e.g. air pollution.

AHRR hypomethylation is a potential biomarker of smoking history and captures former smoking even after more than 35 years of smoking cessation[22]. Self-reported smoking status and cumulative smoking are often insufficiently reported and the latter may be subjected to recall bias[16,23], indicating the need for more objective markers of smoking behavior. Currently, cotinine concentrations in blood or urine or measurements of exhaled CO concentrations, both with biological half-lives below 24 hours, 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 to reflect long-term smoking history[10,22]. The concept of *AHRR* methylation extent as a biomarker of long-term smoking history[17], is supported by our findings that among former smokers, *AHRR* methylation extent 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. Interestingly, 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 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 exposures from cigarette smoke are monitored by *AHRR* hypomethylation. Our observational study can, however, not clarify this important point. *AHRR* hypomethylation has been 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 metabolizing genes such as *CYP1A1*[25]. In turn, this may compromise the capability to metabolize and remove harmful agents such as polyaromatic hydrocarbons, potentially leading to impaired lung function[30]. Alternatively, as *AHRR* deficient mice show less expression of certain proinflammatory molecules after lipopolysaccharide injection[31], this may imply that AHRR is

involved in inflammatory response regulation also included in declining lung function. However, we cannot exclude that for intragenic methylation such as *AHRR* cg05575921, methylation changes 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.

Interestingly, in our study we found several sex differences. Firstly, in all smoking strata, men had lower methylation extent than women in models adjusted for age and height. This may be due to differences in smoking behavior and general environmental exposures between men and women, or could reflect a higher susceptibility to tobacco-induced DNA alterations in men. Secondly, in women, the methylation extent did not discriminate well between former and never smokers and was not associated with respiratory symptoms among former smokers. Lastly, in prospective analyses of women, low methylation extent was not associated with a steeper lung function decline in any smoking strata. Differences in reporting of smoking behavior among men and women could be one explanation, 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 has been 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 higher risk of respiratory symptoms.

Strengths of our study include a large sample size from a homogenous population and repeated measurements of lung function. Still, some potential limitations should be considered. First, bias may be introduced in the use of repeated spirometry; since *AHRR* hypomethylation is associated with lung function and all-cause mortality[17], individuals with low methylation extent may not attend both examinations due to morbidity or death as also 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. Additionally, since our study population consist solely of Danes, our findings may not necessarily be applicable to individuals of different ethnicity, 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 higher risk of respiratory symptoms. Our results extend the number of smoking-related phenotypes associated with *AHRR* hypomethylation and strengthen the evidence of *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.

References

1 Egger G, Liang G, Aparicio A *et al.* Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004; 429: 457-63.

2 van der Maarel SM. Epigenetic mechanisms in health and disease. Ann Rheum Dis 2008; 67 Suppl 3: iii97-100.

3 Alegria-Torres JA, Baccarelli A, Bollati V. Epigenetics and lifestyle. Epigenomics 2011; 3: 267-77.

4 Zeilinger S, Kuhnel B, Klopp N *et al.* Tobacco smoking leads to extensive genome-wide changes in DNA methylation. PLoS One 2013; 8: e63812.

5 Fasanelli F, Baglietto L, Ponzi E *et al.* Hypomethylation of smoking-related genes is associated with future lung cancer in four prospective cohorts. Nat Commun 2015; 6: 10192.

6 Joehanes R, Just AC, Marioni RE *et al.* Epigenetic Signatures of Cigarette Smoking. Circ Cardiovasc Genet 2016.

7 Zhang Y, Breitling LP, Balavarca Y *et al.* Comparison and combination of blood DNA methylation at smoking-associated genes and at lung cancer-related genes in prediction of lung cancer mortality. Int J Cancer 2016; 139: 2482-92.

8 Elliott HR, Tillin T, McArdle WL *et al.* Differences in smoking associated DNA methylation patterns in South Asians and Europeans. Clin Epigenetics 2014; 6: 4.

9 Dogan MV, Shields B, Cutrona C *et al.* The effect of smoking on DNA methylation of peripheral blood mononuclear cells from African American women. BMC Genomics 2014; 15: 151.

10 Philibert RA, Beach SR, Brody GH. Demethylation of the aryl hydrocarbon receptor repressor as a biomarker for nascent smokers. Epigenetics 2012; 7: 1331-8.

11 Guida F, Sandanger TM, Castagne R *et al.* Dynamics of smoking-induced genome-wide methylation changes with time since smoking cessation. Hum Mol Genet 2015; 24: 2349-59.

12 Reynolds LM, Magid HS, Chi GC *et al.* Secondhand Tobacco Smoke Exposure Associations with DNA Methylation of the Aryl Hydrocarbon Receptor Repressor. Nicotine Tob Res 2016.

13 Joubert BR, Felix JF, Yousefi P *et al.* DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. Am J Hum Genet 2016; 98: 680-96.

14 Willemse BW, Postma DS, Timens W *et al.* The impact of smoking cessation on respiratory symptoms, lung function, airway hyperresponsiveness and inflammation. Eur Respir J 2004; 23: 464-76.

15 Eisner MD, Anthonisen N, Coultas D *et al.* An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2010; 182: 693-718.

16 Shipton D, Tappin DM, Vadiveloo T *et al.* Reliability of self reported smoking status by pregnant women for estimating smoking prevalence: a retrospective, cross sectional study. BMJ 2009; 339: b4347.

17 Bojesen SE, Timpson N, Relton C *et al.* AHRR (cg05575921) hypomethylation marks smoking behaviour, morbidity and mortality. Thorax 2017; 72: 646-53.

18 Juul K, Tybjaerg-Hansen A, Marklund S *et al.* Genetically increased antioxidative protection and decreased chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2006; 173: 858-64.

19 Quanjer PH, Stanojevic S, Cole TJ *et al.* Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. Eur Respir J 2012; 40: 1324-43.

20 Fletcher C, Peto R. The natural history of chronic airflow obstruction. Br Med J 1977; 1: 1645-8.

21 Cheng J, Edwards LJ, Maldonado-Molina MM *et al.* Real longitudinal data analysis for real people: building a good enough mixed model. Stat Med 2010; 29: 504-20.

22 Guida F, Sandanger TM, Castagne R *et al.* Dynamics of smoking-induced genome-wide methylation changes with time since smoking cessation. Hum Mol Genet 2015; 24: 2349-59.

23 Wilcox RG, Hughes J, Roland J. Verification of smoking history in patients after infarction using urinary nicotine and cotinine measurements. Br Med J 1979; 2: 1026-8.

24 Cuozzo C, Porcellini A, Angrisano T *et al.* DNA damage, homology-directed repair, and DNA methylation. PLoS Genet 2007; 3: e110.

25 Lee KW, Pausova Z. Cigarette smoking and DNA methylation. Front Genet 2013; 4: 132.

26 Satta R, Maloku E, Zhubi A *et al.* Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. Proc Natl Acad Sci U S A 2008; 105: 16356-61.

27 Reynolds LM, Wan M, Ding J *et al.* DNA Methylation of the Aryl Hydrocarbon Receptor Repressor Associations With Cigarette Smoking and Subclinical Atherosclerosis. Circ Cardiovasc Genet 2015; 8: 707-16.

28 Monick MM, Beach SR, Plume J *et al.* Coordinated changes in AHRR methylation in lymphoblasts and pulmonary macrophages from smokers. Am J Med Genet B Neuropsychiatr Genet 2012; 159B: 141-51.

29 Haarmann-Stemmann T, Abel J. The arylhydrocarbon receptor repressor (AhRR): structure, expression, and function. Biol Chem 2006; 387: 1195-9.

30 Zhou Y, Sun H, Xie J *et al.* Urinary Polycyclic Aromatic Hydrocarbon Metabolites and Altered Lung Function in Wuhan, China. Am J Respir Crit Care Med 2016; 193: 835-46.

31 Brandstatter O, Schanz O, Vorac J *et al.* Balancing intestinal and systemic inflammation through cell type-specific expression of the aryl hydrocarbon receptor repressor. Sci Rep 2016; 6: 26091.

32 Jjingo D, Conley AB, Yi SV *et al.* On the presence and role of human gene-body DNA methylation. Oncotarget 2012; 3: 462-74.

Tables

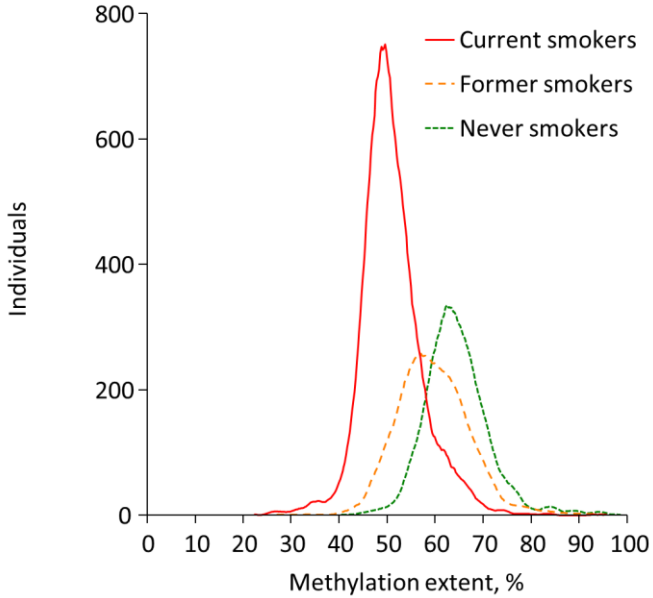
Table 1. Differences in baseline characteristics of individuals according to tertiles of *AHRR* methylation extent and smoking status.

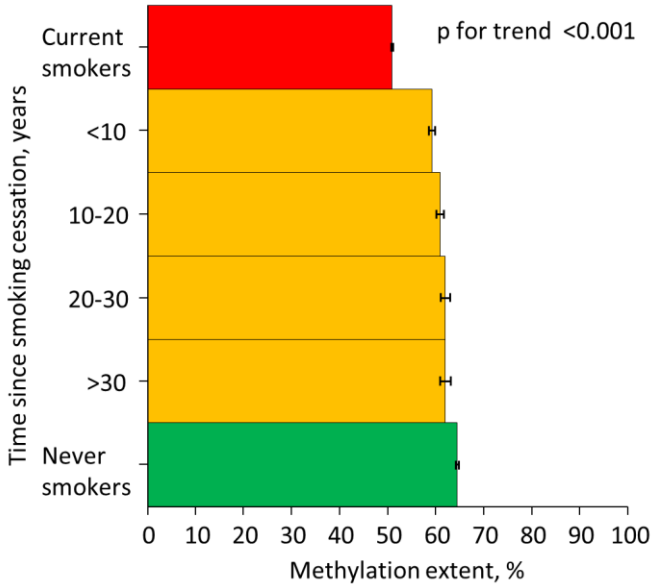
		Methylation extent tertiles			Smoking status		
Characteristic	All	Lowest	Middle	Highest	Current smokers	Former smokers	Never smokers
Individuals	9113	3058	3053	3002	4426	2356	2331
Methylation extent, %	56 (50-63)	48 (46-50)	56 (54-58)	66 (63-69)	50 (47-54)	59 (54-64)	64 (60-68)
Men	4065 (45)	1580 (52)	1445 (47)	1040 (35)	2112 (48)	1209 (51)	744 (32)
Age	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 a higher education	1837 (20)	435 (14)	641 (21)	761 (25)	707 (16)	568 (24)	562 (24)
Body mass index, kg/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[a]	26 (14-40)	32 (21-45)	25 (13-40)	13 (5.0-25)	30 (18-43)	20 (8.5-35)	0

Data are expressed as number and percentage for categorical values and median and interquartile range for continuous values.

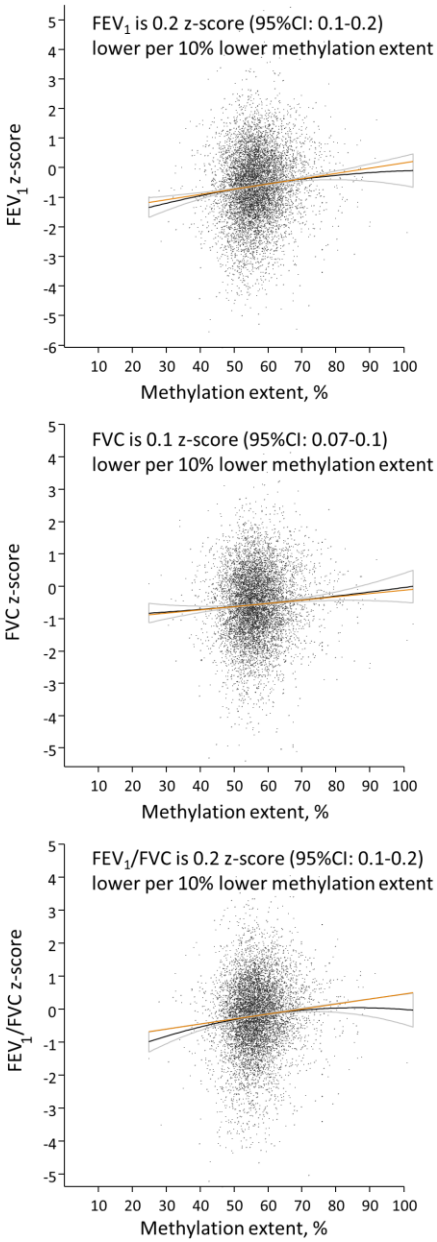
[a] Calculated for current and former smokers only.

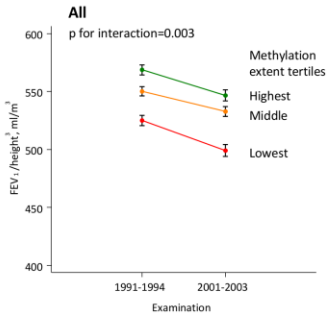
FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.

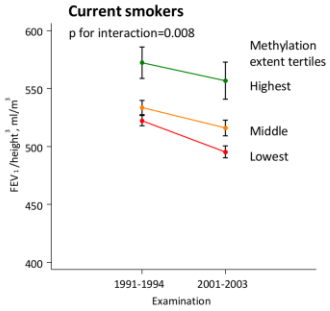

800
600
400
200
0
Individuals
Current smokers
Former smokers
Never smokers
0 10 20 30 40 50 60 70 80 90 100
Methylation extent, %

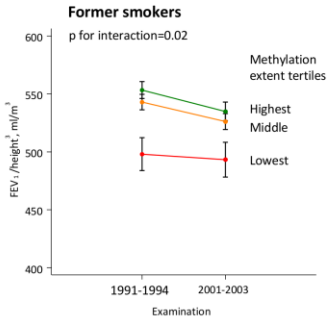

p for trend <0.001
Current smokers
<10
10-20
20-30
>30
Never smokers
Time since smoking cessation, years
0 10 20 30 40 50 60 70 80 90 100
Methylation extent, %

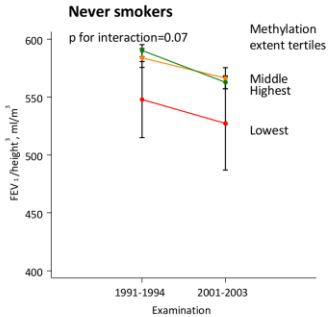
Multivariable + smoking adjusted



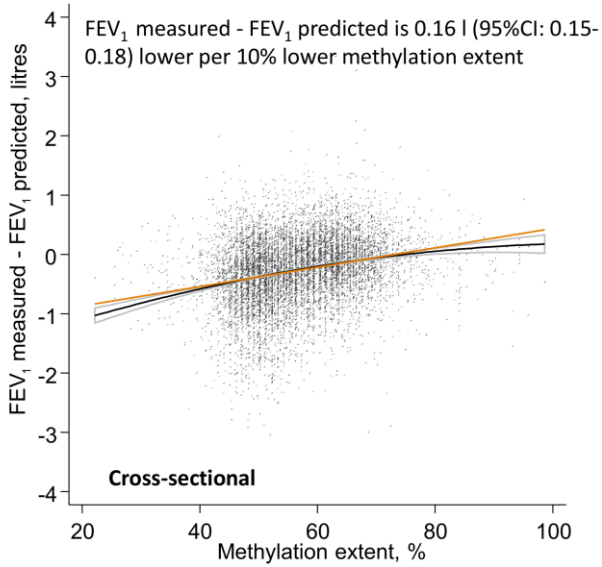

All
p for interaction=0.003
Methylation extent tertiles
Highest
Middle
Lowest
FEV1/height2, ml/m2
600
550
500
450
400
1991-1994
2001-2003
Examination


Current smokers
p for interaction=0.008
Methylation extent tertiles
Highest
Middle
Lowest
FEV1/height2, ml/m2
600
550
500
450
400
1991-1994
2001-2003
Examination

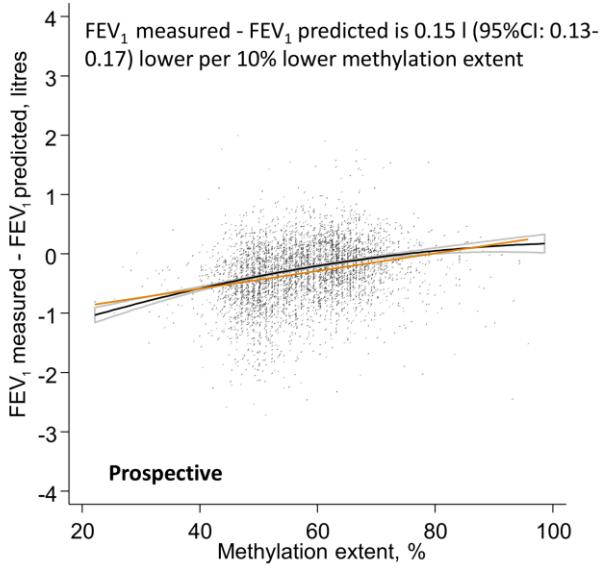

Former smokers
p for interaction=0.02
Methylation extent tertiles
Highest
Middle
Lowest
FEV1/height2, ml/m2
600
550
500
450
400
1991-1994
2001-2003
Examination


Never smokers
p for interaction=0.07
Methylation extent tertiles
Middle
Highest
Lowest
FEV1/height2, ml/m2
600
550
500
450
400
1991-1994
2001-2003
Examination

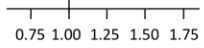
Methylation extent (measured in 1991-1994) and the difference between measured FEV_1 and predicted FEV_1 in the 1991-1994 examination



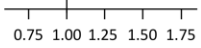
Methylation extent (measured in 1991-1994) and the difference between measured FEV_1 and predicted FEV_1 in the 2001-2003 examination



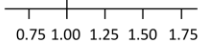
Outcome	Current smokers N/Cases	Current smokers OR (95%CI)	Former smokers N/Cases	Former smokers OR (95%CI)	Never smokers N/Cases	Never smokers OR (95%CI)
Chronic bronchitis	4426/885	1.26 (1.10-1.44)	2356/226	1.29 (1.04-1.60)	2331/120	1.40 (1.04-1.88)
Cough during exercise	4426/970	1.34 (1.17-1.53)	2356/294	1.15 (0.96-1.38)	2331/200	0.95 (0.77-1.19)
Sputum	4426/1450	1.32 (1.18-1.48)	2356/377	1.17 (0.99-1.39)	2331/211	1.14 (0.92-1.42)
Wheezing	4426/1682	1.28 (1.14-1.43)	2356/492	1.20 (1.03-1.40)	2331/291	1.15 (0.94-1.39)
Dyspnea (mMRC≥2)	4426/696	1.21 (1.04-1.41)	2356/384	1.02 (0.86-1.22)	2331/251	0.86 (0.70-1.05)
Any respiratory symptoms	4426/2494	1.34 (1.20-1.49)	2356/874	1.16 (1.02-1.32)	2331/600	0.98 (0.85-1.14)



OR (95%CI) per 10% lower methylation extent



OR (95%CI) per 10% lower methylation extent



OR (95%CI) per 10% lower methylation extent

Supplementary information

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

Jakob B. Kodal[1,2], Camilla J. Kobylecki[1,2], Signe Vedel-Krogh[1,2], Børge G. Nordestgaard[1,2,3], Stig E. Bojesen[1,2,3]*

[1]Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital, DK-2730 Herlev, Denmark

[2]Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

[3]The Copenhagen General Population Study, Herlev and Gentofte Hospital, Copenhagen University Hospital, Denmark

Table of contents — Page number

Methods

Spirometry

Forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were determined using a dry wedge spirometer (Vitalograph; Maids Moreton, Buckinghamshire, UK) at the 1991-1994 and 2001-2003 examinations. Instruments were calibrated daily against a 1 L syringe. For each individual, spirometry was performed in triplicate, and results were only accepted if variation between the two best-performing of these was less than 5%. Spirometry measurements were available for 98.7% of individuals with available methylation measurements. FEV_1, FVC, and FEV_1/FVC z-scores were calculated according to the global lung function initiative 2012 equations (GLI-2012) [20]. Cross-sectional analyses were based on spirometry measurements from the 1991-1994 examination, whereas prospective analyses also included spirometry measurements from the 2001-2003 examination.

Covariates

Information on 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 meters squared.

Information on smoking status was self-reported at the 1991-1994 and 2001-2003 examinations. Self-reported smoking of cigarettes, cheroots, cigars, and pipe tobacco were recalculated into daily grams of tobacco consumption. Cumulative smoking was calculated for former and current smokers in pack-years; a pack-year was defined as 20 cigarettes or equivalent per day smoked for one year. We did not have information on traffic air pollution exposure.

Information on COPD hospitalizations (ICD8: 491-492, ICD10:J41-J44) and death was obtained from the national Danish Patient Registry and the national Danish Civil Registration System.

Respiratory symptoms

All included respiratory symptoms were self-reported from the 1991-1994 examination. Chronic bronchitis was defined as coughing up sputum (in the morning or during the day) for three consecutive months every year. Exercise induced cough was defined as occasional cough during exercise. Sputum was defined as coughing up sputum in the morning and/or during the day. Dyspnea was defined according to the modified Medical Research Council dyspnea scale (mMRC) ≥2, that is, experiencing more shortness of breath than peers, the need to stop to recover breath when walking in one's own pace, and/or experiencing shortness of breath while bathing or getting dressed. Wheezing was defined as whistling or wheezing while breathing, and any respiratory symptom was defined as answering affirmative to any of the above questions.

Supplementary Table S1: Association between *AHRR* methylation extent and age, sex and height according to smoking status.

	AHRR methylation extent			
	N	β	95%CI	R^2
Never smokers				
Age, per 10 year	2331	-0.01	-0.03; 0.002	0.001
Sex, men versus women	2331	-0.96	-1.54; -0.38	0.005
Height, cm	2331	-0.019	-0.47; 0.01	0.001
Age+ sex+ height multivariate model				0.08
Age, per 10 year	2331	-0.02	-0.04; -0.004	
Sex, men versus women	2331	-1.21	-2.01; -0.41	
Height, cm	2331	0.002	-0.04; 0.04	
Current smokers				
Age, per 10 year	4426	0.02	0.01; 0.04	0.003
Sex, men versus women	4426	-0.62	-1.0; -0.24	0.002
Height, cm	4426	-0.002	-0.02; 0.02	0.000
Age+ sex+ height multivariate model				0.009
Age, per 10 year		0.04	0.02; 0.05	
Sex, men versus women		-1.4	-1.9; -0.9	
Height, cm		0.07	0.04; 0.10	
Former smokers				
Age, per 10 year	2356	-0.03	-0.05; -0.008	0.003
Sex, men versus women	2356	-3.1	-3.7; -2.5	0.04
Height, cm	2356	-0.09	-0.1; -0.06	0.01
Age+ sex+ height multivariate model				0.05
Age, per 10 year		-0.02	-0.04; 0.0003	
Sex, men versus women		-3.3	-4.1; -2.5	
Height, cm		0.02	-0.03; 0.07	

Associations are all based on linear models.
CI: confidence interval; N: number of individuals; R^2: coefficient of determination; β: Regression coefficient.

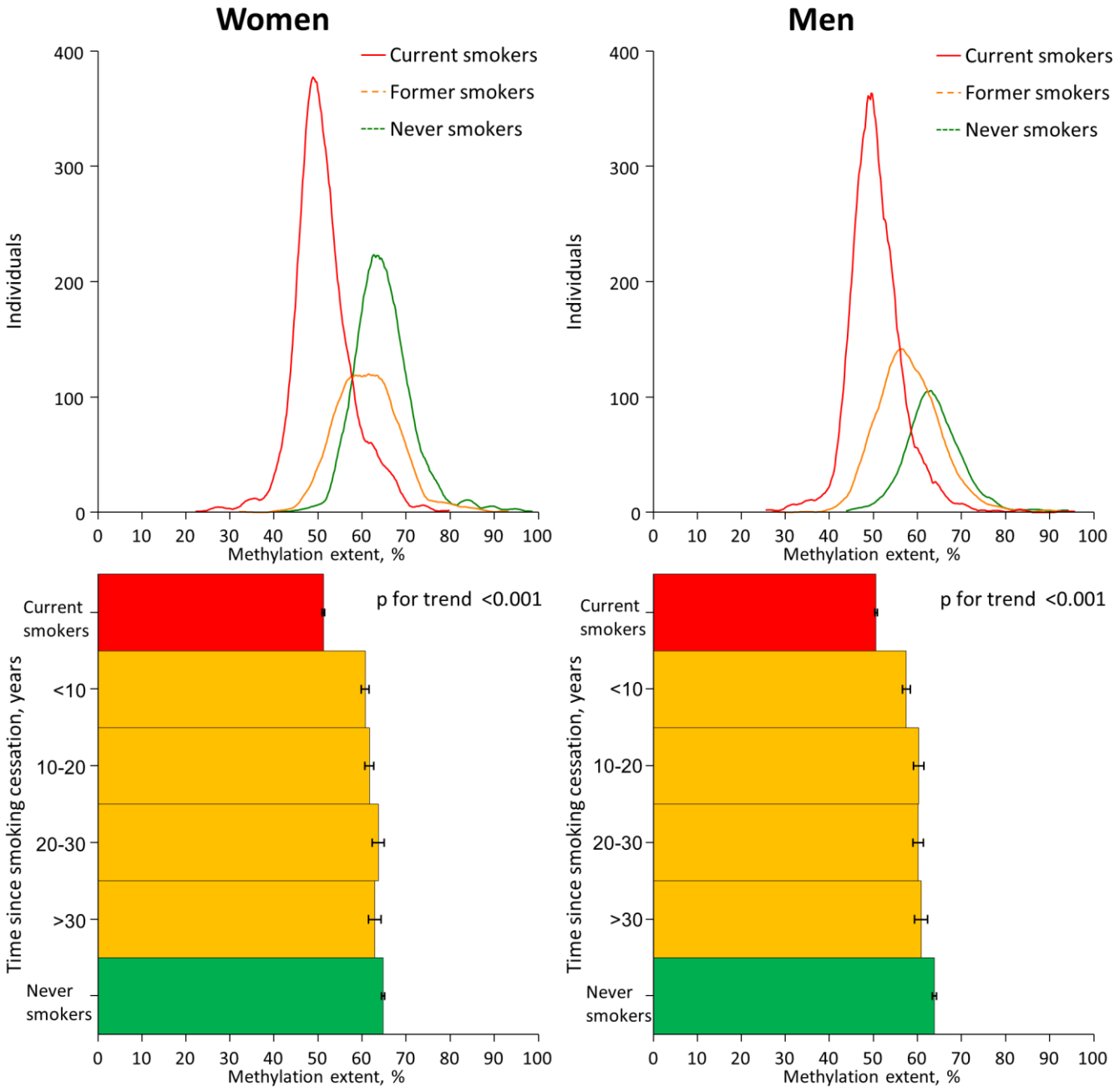
Supplementary Table S2. Characteristics of individuals according to examination attendance.

Characteristic	All	Individuals attending both examinations	Individuals only attending the 1991-1994 examination	p-value
Individuals	9113	4532	4581	<0.001
AHRR Methylation extent, %	56 (50 to 63)	57 (50 to 64)	54 (49 to 61)	<0.001
Men	4065 (45)	1924 (42)	2141 (47)	<0.001
Age	60 (47 to 70)	54 (43 to 64)	66 (54 to 73)	<0.001
Sputum	1231 (14)	429 (9.5)	802 (18)	<0.001
Any respiratory symptoms	4032 (44)	1618 (36)	2350 (51)	<0.001
COPD related hospital contact[a]	678 (8.0)	235 (5.2)	443 (9.7)	<0.001
Dead within 15 years[b]	3143 (35)	462 (10)	2681 (59)	<0.001
Never smokers	2331 (26)	1359 (30)	972 (21)	<0.001
Current smokers	4426 (49)	1961 (43)	2465 (54)	<0.001
Former smokers	2356 (26)	1212 (27)	1144 (25)	0.05
Exposed to passive smoking	3283 (36)	1661 (37)	1622 (35)	0.22
Dust and fumes exposure	1689 (19)	712 (16)	977 (21)	<0.001
Pack years[c]	18 (0 to 35)	25 (22 to 27)	25 (23 to 28)	<0.001
FEV_1 z-score	-0.55 (-1.4 to 0.26)	-0.27 (-1.0 to 0.46)	-0.85 (-1.8 to -0.035)	<0.001
FEV_1/FVC	0.79 (0.73 to 0.83)	0.80 (0.75 to 0.84)	0.77 (0.70 to 0.82)	<0.001

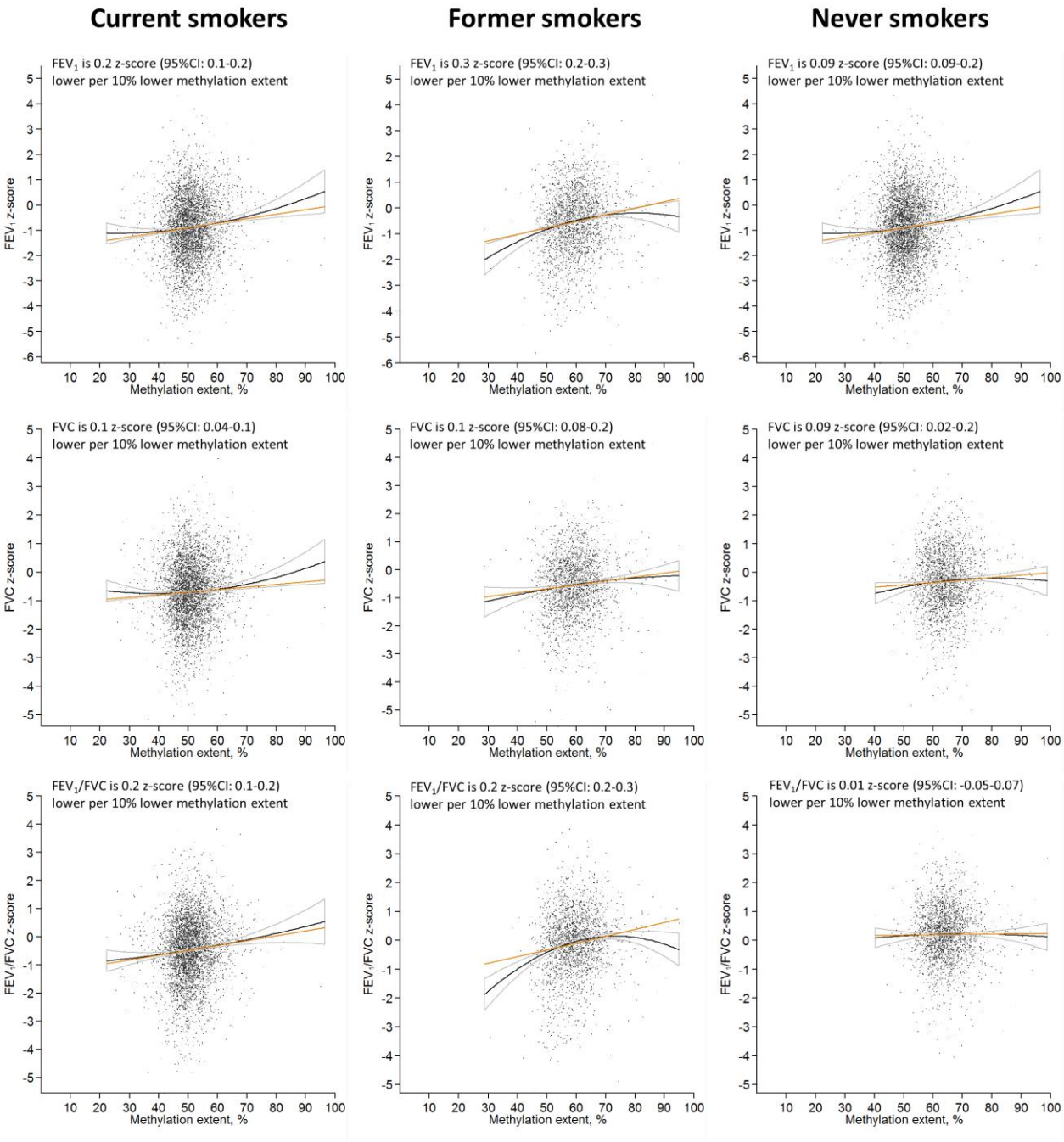
Data are expressed as number and percentage for categorical values and median and interquartile range for continuous values. P-values were calculated using Cuzick's test for trend for continuous values and Pearson's chi-squared test for dichotomous values.

[a] individuals receiving a COPD diagnosis at a hospital before November 6 2014. [b] individuals who died within 15 years of first examination attendance. [a] and [b] are based on data from national registers with complete follow-up. [c] Calculated for current and former smokers only.

FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.

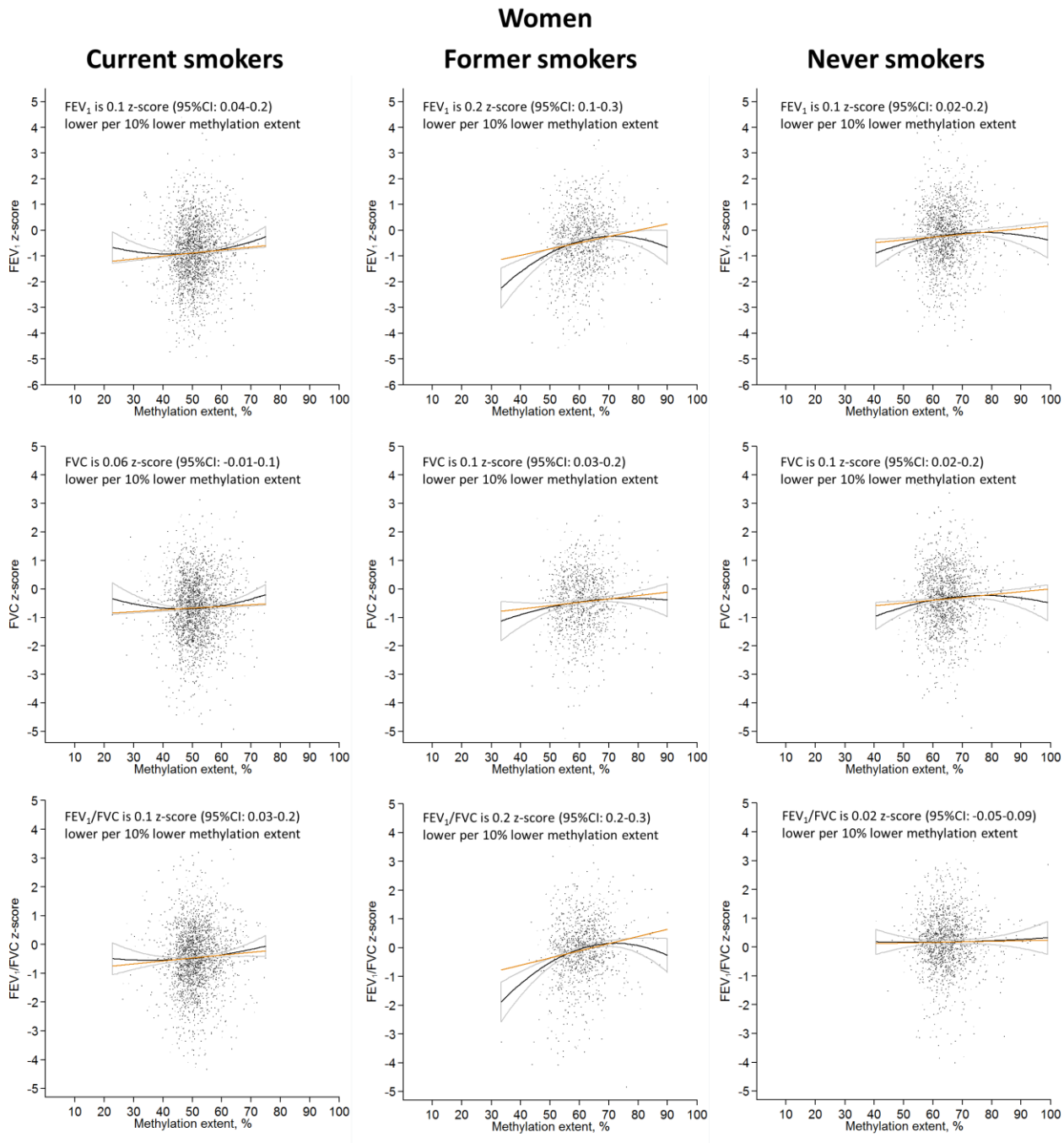


Supplementary Figure S1. Distribution of *AHRR* methylation extent in women and men.
The upper panels show the distribution of methylation extent among current smokers, former smokers, and never smokers for each sex separately. The lower panels show the mean values of methylation extent with 95% confidence intervals for subgroups of former smokers according to years since smoking cessation. P-values for linear trends are reported. Current and never smokers are shown for reference. The black bars represent the 95% confidence interval.



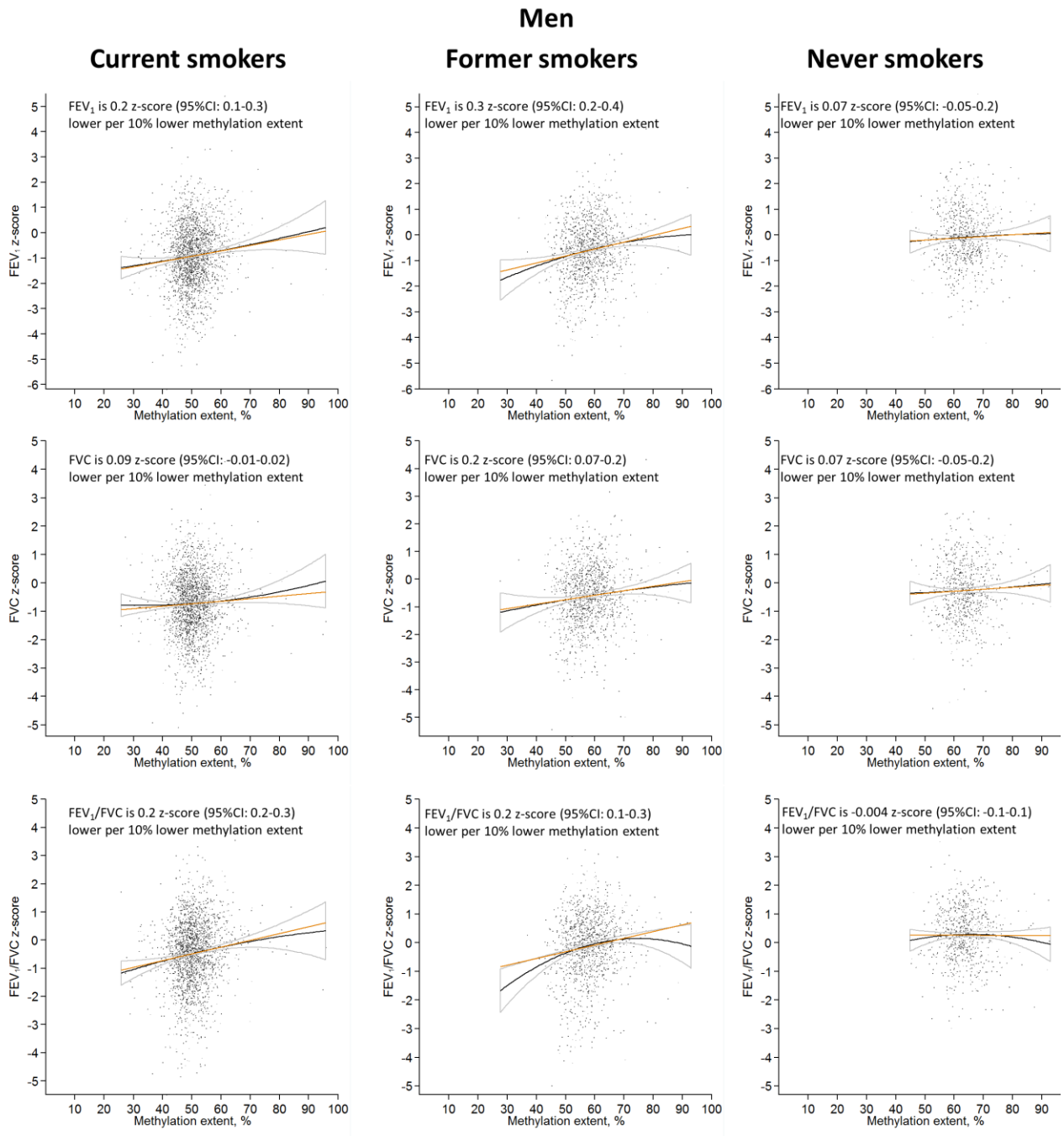
Supplementary Figure S2. Associations of *AHRR* methylation extent with FEV_1 z-score, FVC z-score, and FEV_1/FVC z-score stratified according to smoking status.

All analyses were adjusted for age, sex, body mass index, dust and fume exposure, passive smoking, educational level, 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. CI: confidence interval; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.



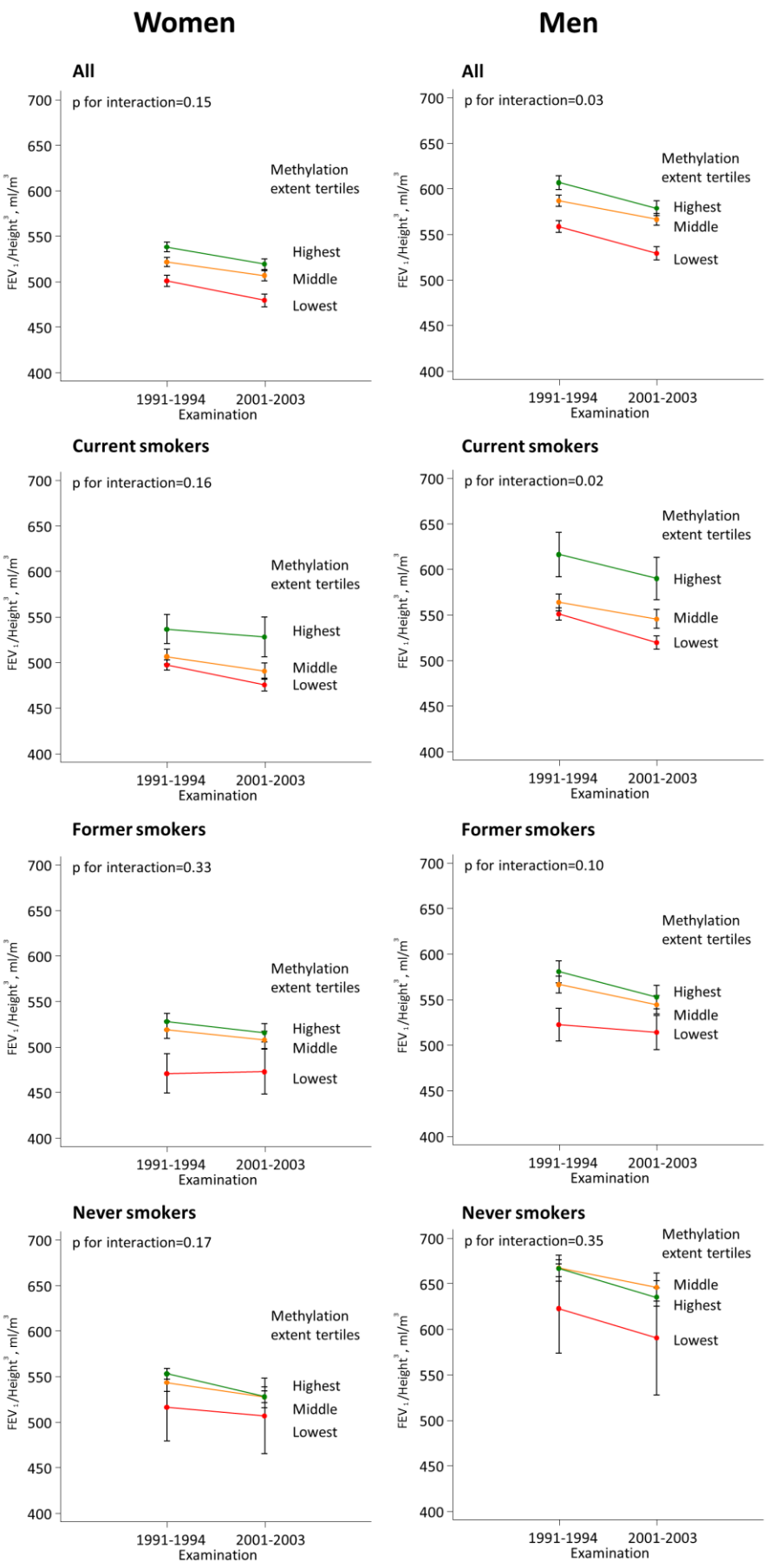
Supplementary Figure S3. Associations of *AHRR* methylation extent with FEV_1 z-score, FVC z-score, and FEV_1/FVC z-score stratified according to smoking status in women.

All analyses were adjusted for age, body mass index, dust and fume exposure, passive smoking, educational level, 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. CI: confidence interval; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.



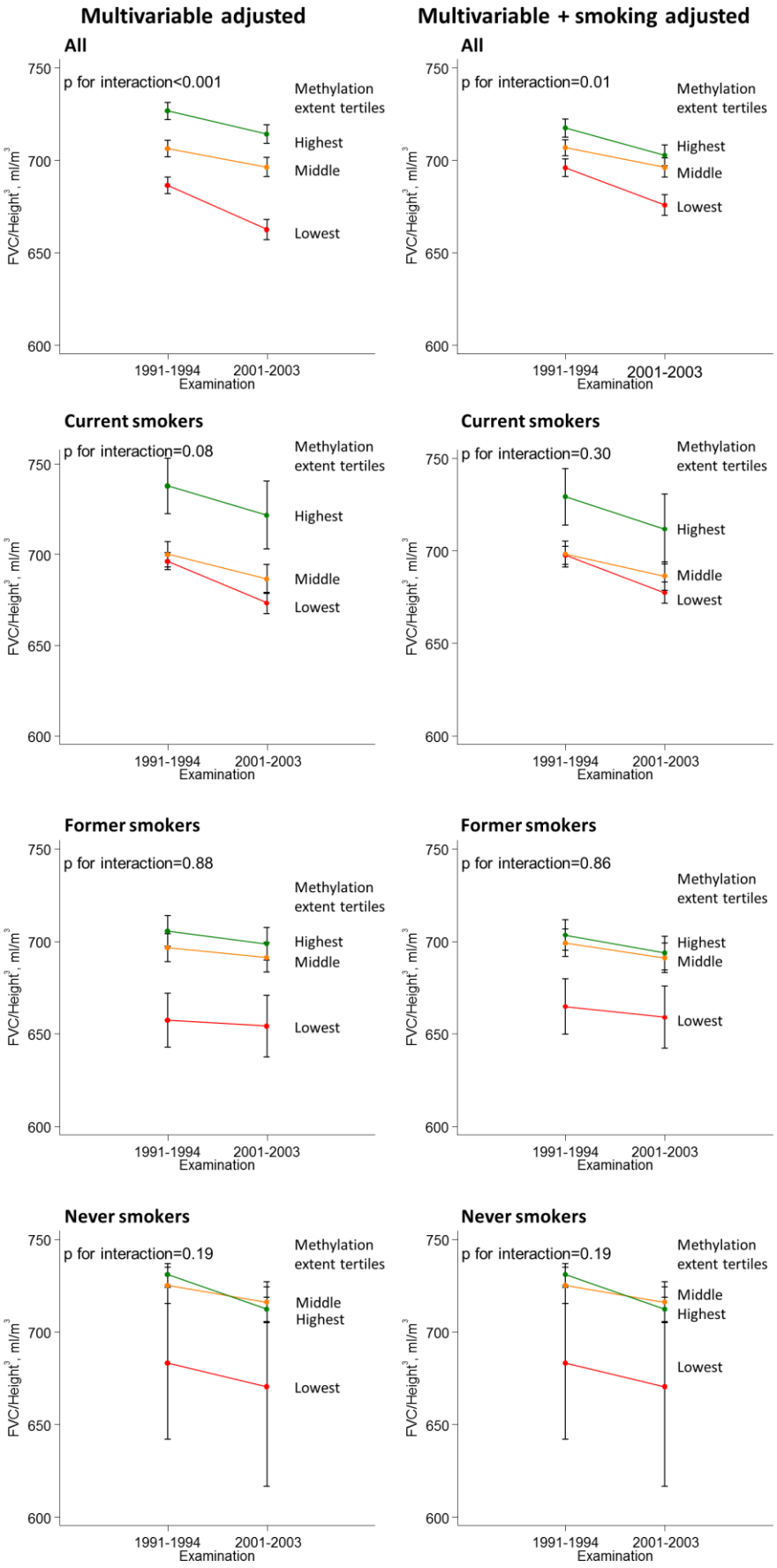
Supplementary Figure S4. Associations of *AHRR* methylation extent with FEV_1 z-score, FVC z-score, and FEV_1/FVC z-score stratified according to smoking status in men.

All analyses were adjusted for age, body mass index, dust and fume exposure, passive smoking, educational level, 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. CI: confidence interval; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.



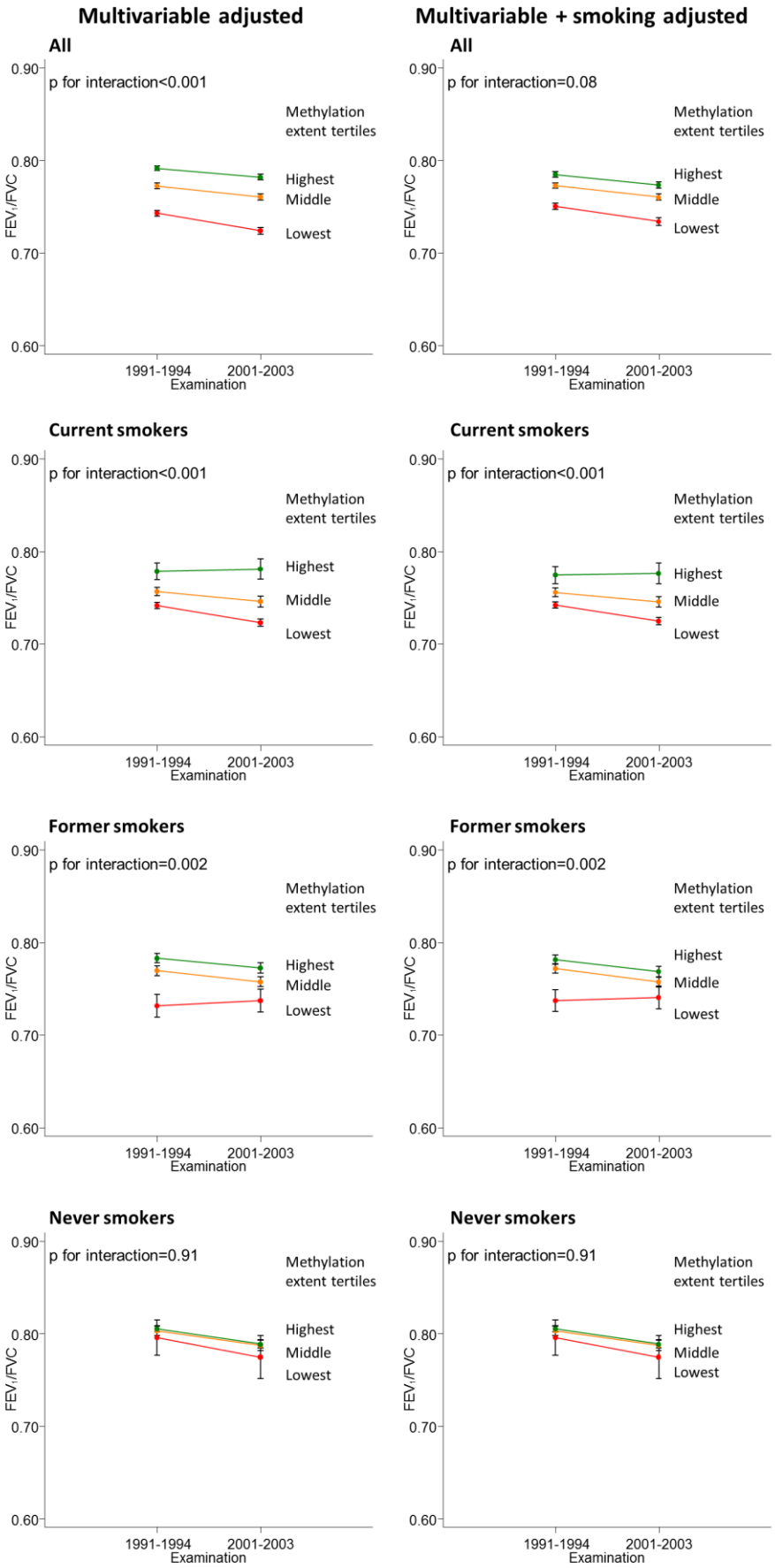
Supplementary Figure S5. Relationship between changes in FEV_1/height^3 according to tertiles of *AHRR* methylation for each sex, overall and stratified according to smoking status.

Analyses were adjusted for cumulative smoking updated at each examination, age, and examination. 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. Identity of each individual was introduced as a random effect to specify the grouping structure, hereby accounting for within-subject correlation. Only baseline height was used. The black bars represent the 95% confidence interval. 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.



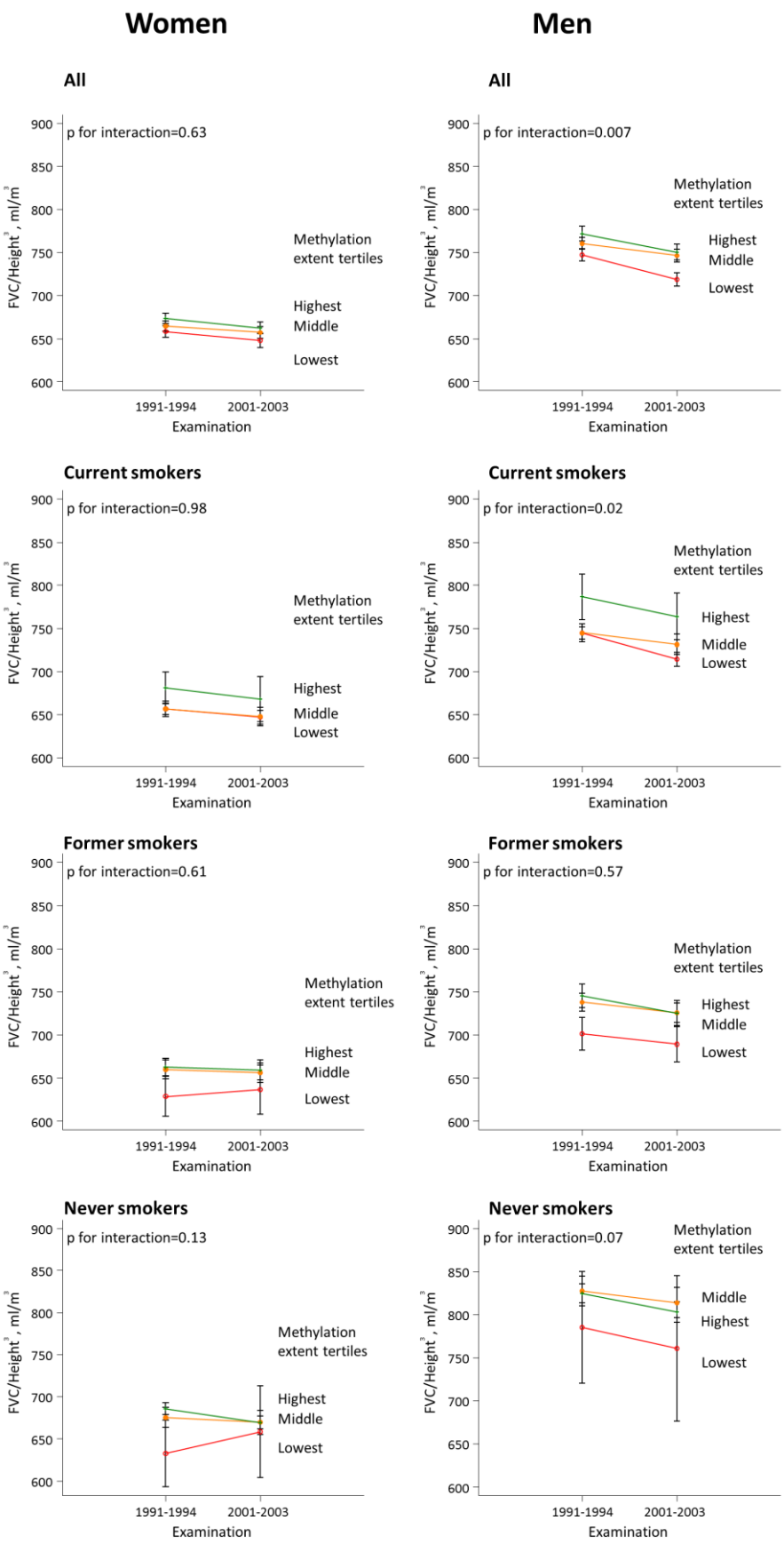
Supplementary Figure S6. Relationship between changes in FVC/height^3 according to tertiles of *AHRR* methylation extent and stratified according to smoking status.

Analyses were adjusted for age, sex, and examination. Multivariable + smoking were further adjusted for cumulative smoking updated at each examination. 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. Identity of each individual was introduced as a random effect to specify the grouping structure, hereby accounting for within-subject correlation. The black bars represent the 95% confidence interval. P-values for interaction of examination (1991-1994 versus 2001-2003) with tertiles of methylation extent are reported. FVC: forced vital capacity.

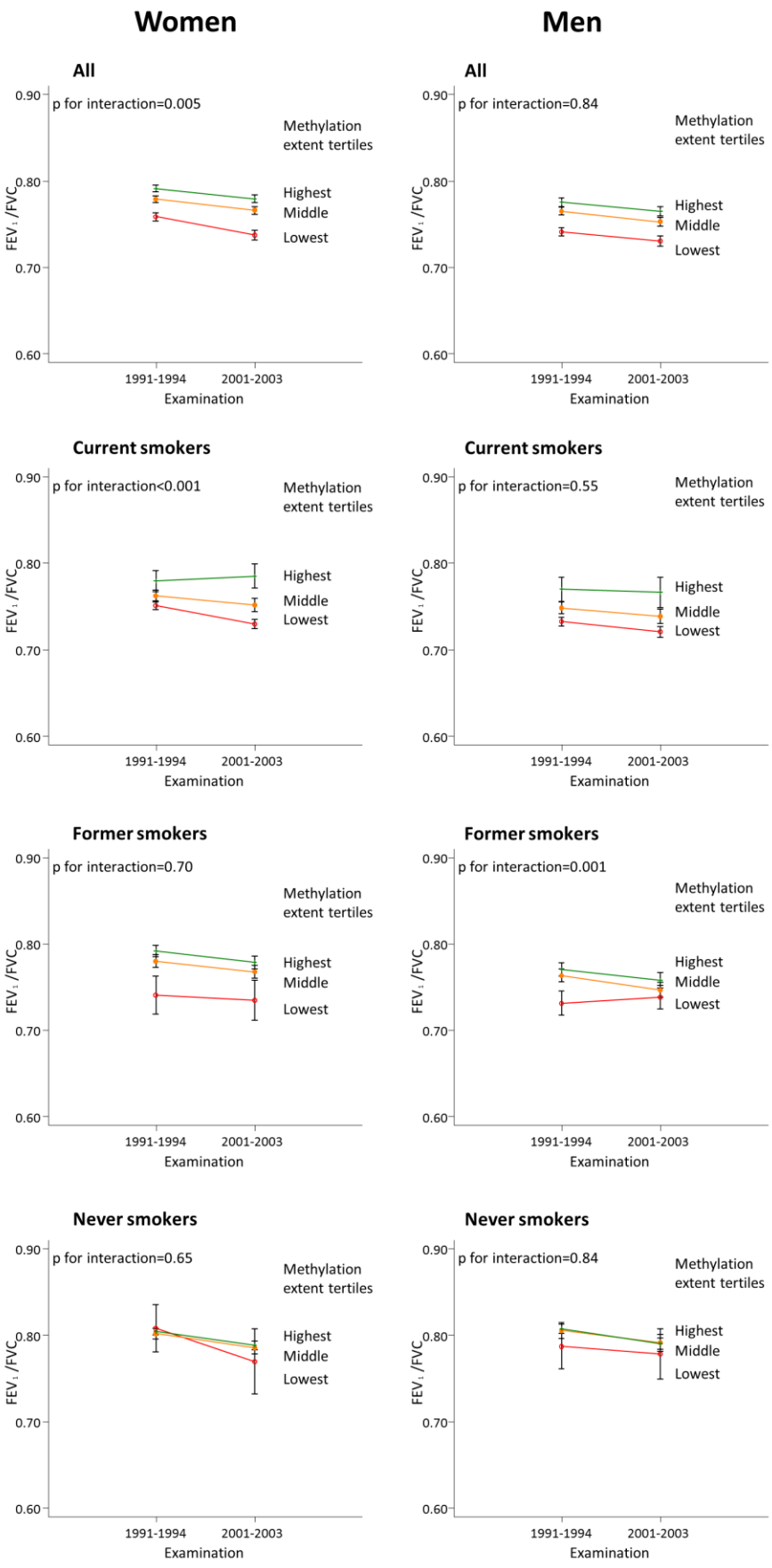


Supplementary Figure S7. Relationship between changes in FEV_1/FVC according to tertiles of *AHRR* methylation extent and stratified according to smoking status.

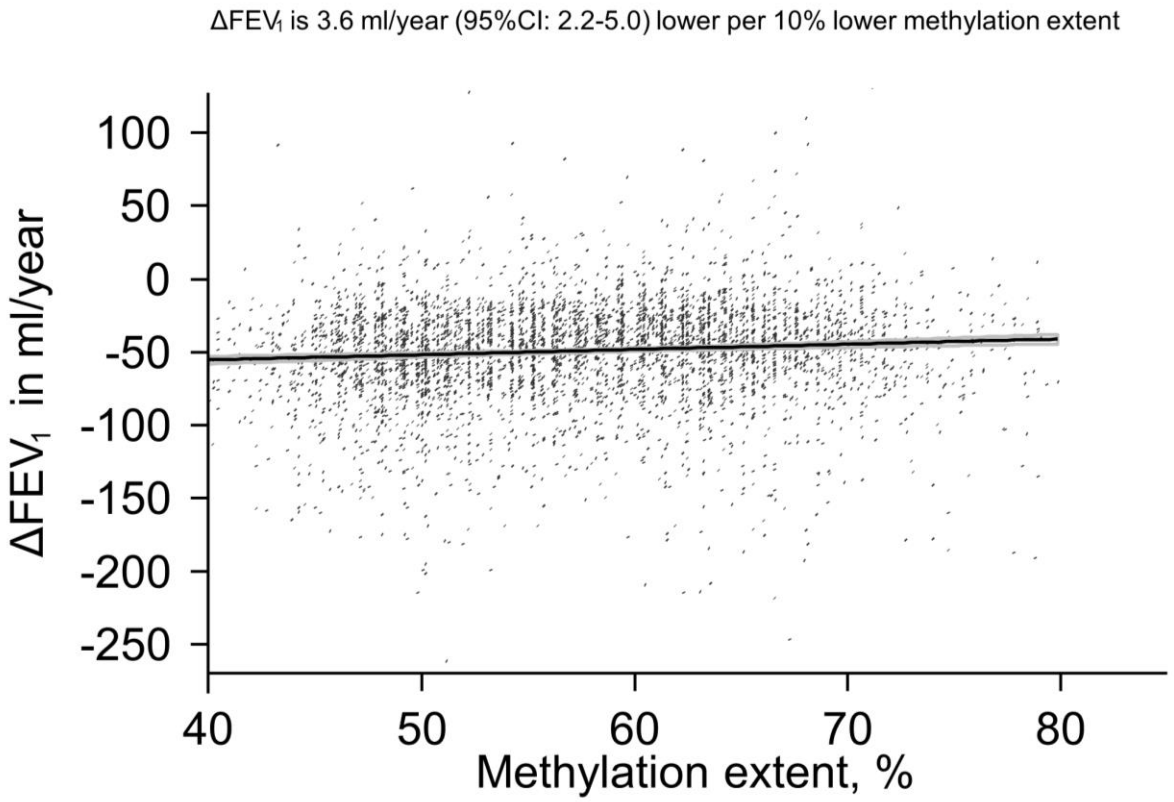
Analyses were adjusted for age, sex, and examination. Multivariable + smoking were further adjusted for cumulative smoking updated at each examination. Based on one to two spirometries for each of 9113 individuals, spanning up to 11.5 years; those individuals with only one measurement were included to increase precision of the baseline estimate. Identity of each individual was introduced as a random effect to specify the grouping structure, hereby accounting for within-subject correlation. The black bars represent the 95% confidence interval. 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; FVC: forced vital capacity.



Supplementary Figure S8. Relationship between changes in FVC/height^3 according to tertiles of *AHRR* methylation for each sex, overall and stratified according to smoking status. Analyses were adjusted for cumulative smoking updated at each examination, age, and examination. 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. Identity of each individual was introduced as a random effect to specify the grouping structure, hereby accounting for within-subject correlation. Only baseline height was used. The black bars represent the 95% confidence interval. P-values for interaction of examination (1991-1994 versus 2001-2003) with tertiles of methylation extent are reported. FVC: forced vital capacity.



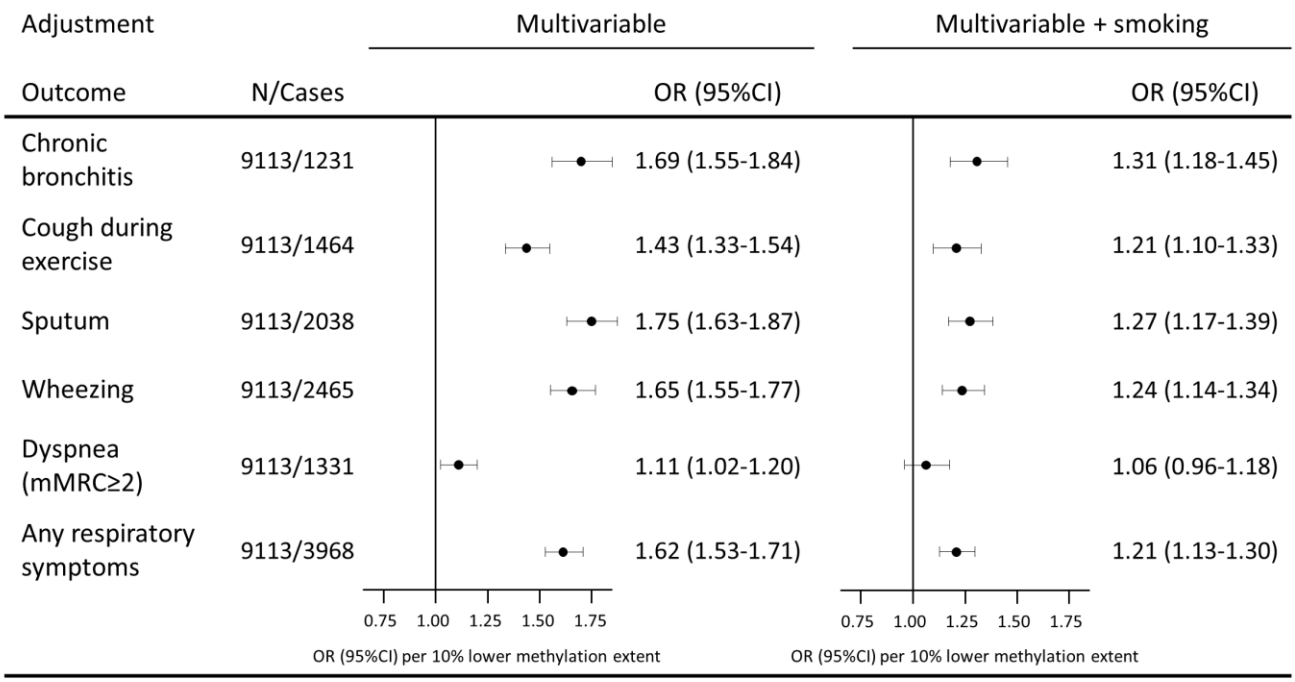
Supplementary Figure S9. Relationship between changes in FEV_1/FVC according to tertiles of *AHRR* methylation for each sex, overall and stratified according to smoking status. Analyses were adjusted for cumulative smoking updated at each examination, age, and examination. Based on one to two spirometries 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. Identity of each individual was introduced as a random effect to specify the grouping structure, hereby accounting for within-subject correlation. Only baseline height was used. The black bars represent the 95% confidence interval. 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; FVC: forced vital capacity.



Supplementary Figure S10. FEV_1 change for individuals attending both examinations according to *AHRR* methylation extent.

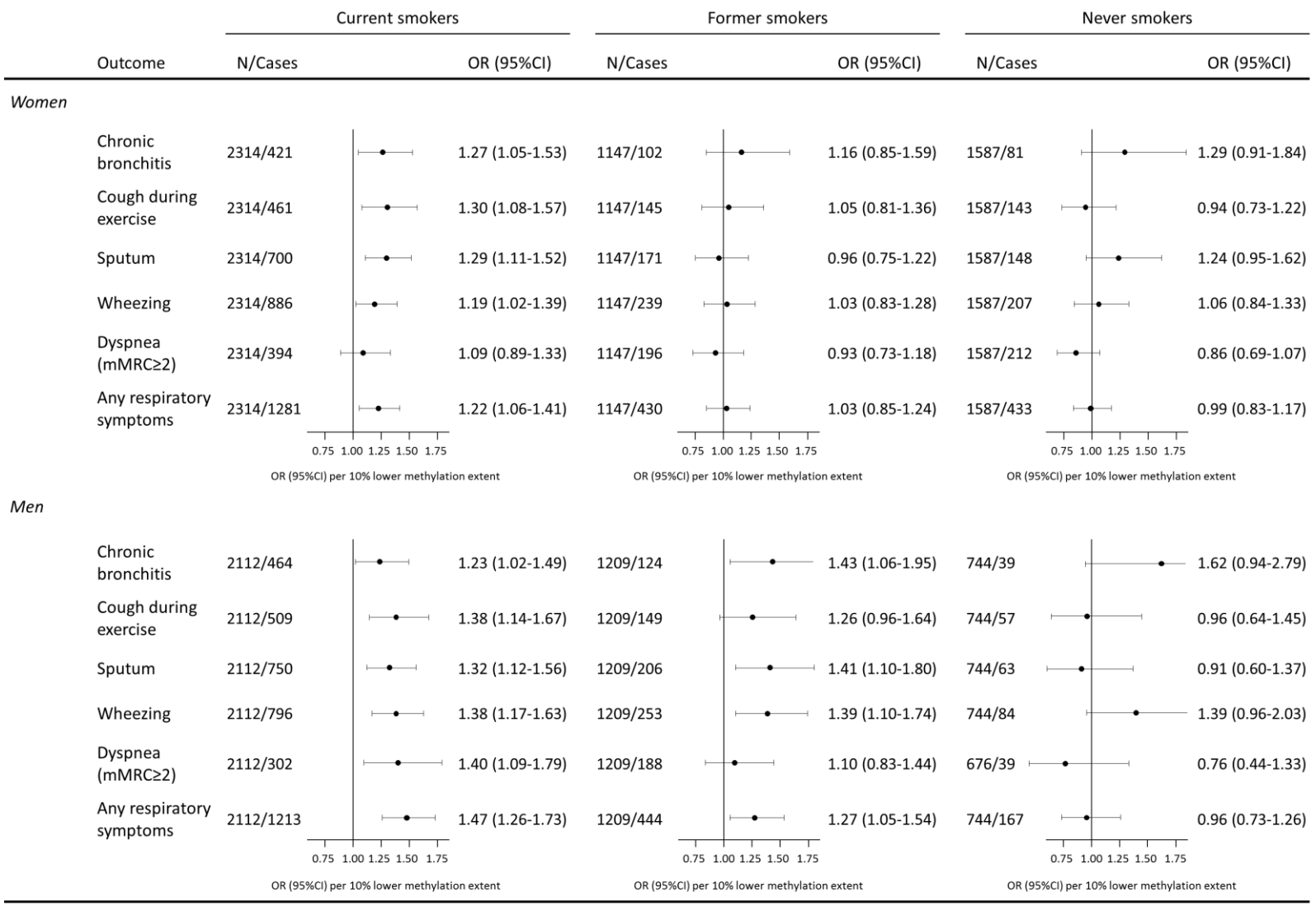
Only individuals participating in both the 1991-1994 and 2001-2003 examinations of the Copenhagen City Heart Study are included. The difference in FEV_1 was then divided by the time in years between each individual's examination dates.

The black line represents an unadjusted linear regression line. The grey area surrounding the regression line represents the 95% confidence interval of the regression line. The black dots represent individual values. CI: confidence interval; FEV_1: forced expiratory volume in 1 s.



Supplementary Figure S11. Odds ratio for respiratory symptoms per 10 % lower *AHRR* methylation extent at the 1991-1994 examination.

Analyses were multivariable adjusted for age, sex, body mass index, dust and fume exposure, passive smoking, educational level, and forced expiratory volume in 1 s z-score. Multivariable + smoking analyses were further adjusted for smoking status and cumulative smoking. CI: confidence interval; mMRC: modified Medical Research Council dyspnea scale; N: number of individuals; OR: odds ratio.



Supplementary Figure S12. Odds ratio for respiratory symptoms per 10 % lower *AHRR* methylation extent stratified by smoking status for each sex separately.

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). CI: confidence interval; mMRC: modified Medical Research Council dyspnea scale; N: number of individuals. OR: odds ratio.