Introduction

Tobacco smoking has been recognized as a risk factor for a variety of complex diseases (CDC 2014), including cardiovascular diseases (CVDs) (Ezzati et al. 2005b), at least 15 types of cancer (Ezzati et al. 2005a), and pulmonary diseases (Decramer et al. 2012). Nevertheless, accurate prediction of smoking-attributable health risk is still hampered by various factors (CDC 2010). In particular, it is well known that self-reported smoking exposure suffers from recall bias or intentional underreporting (Connor Gorber et al. 2009; Rebagliato 2002). Even though a number of biomarkers are well established, such as breath carbon monoxide (CO) and cotinine levels, they exclusively reflect short-term smoking exposure and are of limited use for quantifying cumulative exposure and consequently for predicting smoking-related risk (CDC 2010). DNA or protein adducts are considered integrative biomarkers that reflect internal effective dose of smoking, which may, however, only be useful for carcinogenic risk assessment (CDC 2010; Lodovici and Bigaql 2009). In cardiovascular risk assessment, although several biomarkers have been described and used, no biomarker has yet been identified for specifically predicting smoking-related risk (CDC 2010).

Recent advances in genome-wide methylation profiling have opened new avenues in the search for biomarkers reflecting both current and lifetime smoking exposure that might have the potential to enhance prediction of smoking-related risks. Recently, a number of novel smoking-associated blood DNA methylation biomarkers were identified by using the Infinium HumanMethylation Illumina 450K BeadChip (Joubert et al. 2013a; Zeilinger et al. 2013). Among which seven loci located in four intragenic or intergenic regions (including F2RL3 (cg03636183), AHRR (cg21611338 and cg05575921), 2q37.1 (cg21566642, cg01940273, and cg05951221), 6p21.33 (cg06126421)) were the top seven CpGs reported by both epigenome-wide studies conducted in adults (Shenker et al. 2013a; Zeilinger et al. 2013). To further explore the use of methylation levels of these regions for quantifying biologically effective smoking exposure and for enhancing risk prediction of smoking-related disease, we carried out comprehensive analyses on the associations of methylation at nine CpGs [the top seven CpGs listed above and two other CpGs (AHRR (cg23576855); 2q37.1 (cg06644428)] in those regions reported to be associated with smoking (Shenker et al. 2013a; Zeilinger et al. 2013)] with both current and lifetime smoking exposure as well as mortality in a population-based cohort of older adults. In addition, we aimed to evaluate whether these methylation biomarkers can improve the fatal cardiovascular risk prediction estimated by the Systematic Coronary Risk Evaluation (SCORE) chart of the European Society of Cardiology (Conroy et al. 2003).

Methods

Study design and data collection. The study subjects were selected from the ESTHER study, a statewide population-based cohort study conducted in southwest Germany (Schöttker et al. 2013a). Briefly, 9,949 older adults (50–75 years of age) were enrolled by their general practitioners during a routine health check-up between July 2000 and December 2002, and followed up since then. The distribution of sociodemographic factors and major risk factors in the cohort was similar to the distribution seen in representative surveys of the population in Germany in the corresponding age range (Löw et al. 2004). A genome-wide methylation screen was performed in baseline blood samples.
of 1,000 participants who were recruited between July and October 2000 (i.e., those with the longest follow-up time) and included in the current analysis. The study was approved by the ethics committees of the University of Heidelberg and of the state medical board of Saarland, Germany. Written informed consent was obtained from all participants.

Participants’ sociodemographic characteristics, lifestyle factors, health status, and history of major diseases at baseline were obtained by a standardized self-administered questionnaire. Detailed information on lifetime active smoking was also ascertained from the self-administered questionnaire, including age at initiation of smoking and intensity of smoking at various ages, as well as age of smoking cessation for former smokers. Additional information on height, weight, blood pressure, and prevalent diseases (e.g., diabetes, hypertension, CVD) was extracted from a standardized form completed by the general practitioner during the health check-ups. Prevalent CVD at baseline was defined by either physician-reported coronary heart disease or a self-reported history of myocardial infarction, stroke, pulmonary embolism, or revascularization of coronary arteries. Prevalent cancer [International Classification of Diseases, 10th Revision (ICD-10) codes C00–C99 except nonmelanoma skin cancer (code C44)] was determined by self-report or record linkage with data from the Saarland Cancer Registry [http://www.krebsregister.saarland.de/ziele/ziele1.html (in German)].

Blood samples (21 mL from each participant) were collected during the health check-up and aliquoted and stored at −80°C until further processing. Total cholesterol level was measured in serum by standard high-performance liquid chromatography methods (Schöttker et al. 2013b). Deaths during follow-up (between 2000 and end of 2011) were identified by record linkage with population registries in Saarland; few participants who moved out of Saarland were censored at the date last known to be alive. Information about the major cause of death was obtained from death certificates provided by the local public health offices, and were coded with ICD-10 codes. Cardiovascular and cancer deaths were defined by ICD-10 codes I00–I99 and C00–C99, respectively; nonmelanoma skin cancer (ICD-10 code C44) was excluded.

Methylation assessment. DNA was extracted from whole blood samples collected at baseline by a salting out procedure (Miller et al. 1988) and was allocated in the 96-well format. Three random duplicate samples were placed on each plate as quality controls. The Infinium HumanMethylation450K BeadChip Assay (Illumina Inc., San Diego, CA, USA) was used to quantify DNA methylation at 485,577 CpG sites. Briefly, a sample of 1.5 μg genomic DNA was bisulfite converted, and 200 ng bisulfite-treated DNA was applied to the 450K BeadChips. The samples were analyzed following the manufacturer’s instruction at the Genomics and Proteomics Core Facility of German Cancer Research Center. GenomeStudio® (version 2011.1; Illumina Inc.) was used to extract DNA methylation signals from the scanned arrays (module version 1.9.0; Illumina Inc.) and to calculate methylation intensity (β-value) as a ratio of the methylated signal over the sum of the methylated and unmethylated signals at each CpG according to the manufacturer’s guide, without additional background correction. Data were normalized to internal controls provided by Illumina (Illumina normalization). Methylation intensities at the nine CpGs were extracted from the 450K data.

Statistical analysis. Median methylation intensities at the nine CpGs were determined for strata of sociodemographic characteristics, lifestyle factors, and prevalent diseases; differences in methylation intensities between strata were examined by Kruskal–Wallis tests. Correlations between methylation intensity at the nine CpGs were assessed by Spearman rank correlation coefficients. The associations between smoking indicators (including smoking status, current intensity of smoking, cumulative dose of smoking, and time since cessation of smoking) and methylation intensity at the nine CpGs were assessed by linear regression models, controlling for batch effect, age (years), sex, body mass index (BMI; < 25, 25.0 to < 30.0, ≥ 30.0 kg/m²), physical activity (inactive, insufficient, sufficient), and prevalence of CVD (ICD-10 codes I20–I25, I60–I69), diabetes (ICD-10 codes C10–C14), and cancer (ICD-10 codes C00–C09 except C44) at baseline (model 3). Methylation intensity was entered into the models either as a categorical variable (using the highest quartiles as reference level) or as a continuous variable [calculating hazard ratios (HR) for a decrease in methylation intensity by one standard deviation]. In parallel, the associations between smoking at baseline and all-cause mortality were also estimated by Cox regression, with and without controlling for methylation intensities to explore the role of DNA methylation in smoking-related mortality. The proportional hazards assumption was assessed by martingale-based residuals (Lin et al. 1993). These preliminary analyses showed methylation at two of the nine CpGs (cg05575921, cg06126421) to be most strongly associated with all-cause mortality, whereas much less strong or nonsignificant associations were observed for the other seven CpGs. Additional preliminary analyses were conducted by L1-penalized Cox model (Benner et al. 2010; Goeman 2010) with nine CpGs and other risk factors as covariates; in that model, only cg05575921 and cg06126421 were selected among the nine CpGs. We therefore carried out analyses on all-cause and cause-specific mortality, including CVD, cancer, and other mortality, using a methylation-based score developed according to these two CpGs. Categories of the score were 2, 1, and 0 for participants in the lowest quartiles of both CpGs, in one of the two CpGs, and none of the two CpGs, respectively. In addition, the analyses were repeated after joint classification of participants according to both methylation score and sex.

To further assess the potential contributions of the smoking-associated CpGs for fatal cardiovascular risk prediction, methylation intensity at the nine CpGs individually and jointly added to a Cox regression model consisting of variables of the SCORE (Conroy et al. 2003), including age (years), sex, systolic blood pressure (millimeters of mercury), current smoking (yes, no), and total cholesterol (milligrams per deciliter) and using cardiovascular mortality as the dependent variable, additionally controlling for batch effect. Model fit was compared using Akaike information criterion (AIC) and likelihood ratio (LR) tests. Discrimination of the models was evaluated by Harrell’s C-statistics (Harrell et al. 1996), and the overoptimism was corrected using .632 bootstrap analysis with 1,000 replications [for this purpose, a SAS Macro was adapted from Miao’s work (Miao et al. 2013)]. Bootstrapping is a well-established approach for validation of a predictive model through quantifying the degradation in model predictive accuracy when applied in different data sources, which
is known as overoptimism. The improvement in model performance by adding methylation intensity was examined by both net reclassification improvement (NRI) and integrated discrimination improvement (IDI). The NRI assesses whether participants are classified into clinically relevant risk categories by adding a new factor (e.g., methylation marker) to the risk prediction model (e.g., SCORE model). Absolute risk predictions were first calculated by Cox regression model with and without methylation marker for each individual, followed by assigning risk categories according to the recommended 10-year risk categories: 0–5%, > 5–10%, > 10–20%, and > 20% of predicted probability for a cardiovascular event (Cook 2007; Pencina et al. 2008). Movements are considered separately for cases (deaths) and controls (survivors), and deemed as correct direction if cases move into a higher risk category and controls move into a lower risk category. NRI = [(no. of cases up – no. of cases down)/no. of cases] – [(no. of controls up – no. of controls down)/no. of controls]. IDI estimates the mean difference in predicted probability for cases and controls over all possible cut-off points between models with and without methylation marker (Cook 2010; Pencina et al. 2008). Calibration of all assessed models was examined by May–Hosmer’s simplification of the Gronnesby–Borgan test (May and Hosmer 2004). The study population was divided into five subgroups according to the quintiles of the ranks based on their estimated risk probability, and model calibration was deemed satisfactory if p-values were > 0.05 for comparison of the observed and expected cases in each subgroup. Potential multicollinearity when simultaneously adding both CpGs in the model was assessed by variance inflation factor (VIF) and tolerance values, which did not indicate any relevant multicollinearity (e.g., VIF = 1.46 and tolerance = 0.69 when adding cg05575921 and cg06126421). Sensitivity analyses were carried out by excluding participants with prevalent CVD at baseline (n = 29).

The penalized Cox regression analyses were conducted using the R package “penalized” (version 0.9–42; Goeman et al. 2014), and all other analyses were carried out in SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

## Results

Of 1,000 participants included in the present analysis, mortality follow-up was available for 999 subjects. Of the nine CpG sites assays, cg21566642, cg23576855, and cg21161138 had 3, 1, and 1 missing values, respectively; all other CpGs had complete data. Characteristics of the study population at baseline are shown in Table 1. Equal numbers of men and women of German nationality were included. The mean age was 62 years, and 33.9% of participants were younger than 60 years. More than half of the participants had ever smoked, and 19% still smoked at the time of recruitment, among whom male (61.3%) and younger (< 60 years, 45.2%) participants were somewhat overrepresented. During a median follow-up time of 10.3 years, 143 participants died. Among 135 participants with death certificates (94.4%), 50 died from CVD, 49 died from cancer, and 36 died from other diseases.

### Methylation intensities by demographic and behavioral factors

Methylation intensities across various strata of characteristics of the study population are shown in Table 1 for AHRR cg05575921 and 6p21.33 cg06126421 (see Supplemental Material, Table S1, for all other CpGs). Men had lower methylation intensities than women at all nine CpG sites (all p < 0.0001). Methylation was not significantly associated with age (p > 0.05) except at 2q37.1 cg06644428 (p = 0.0001). Major differences were observed between never, former, and current smokers. Methylation levels for all nine CpGs were lower in current smokers than in never smokers and intermediate in former smokers, and all of the differences across the three group were statistically significant (p < 0.0001).

### Correlations of methylation intensities at the nine CpGs

Mutual Spearman correlation coefficients for methylation intensities at all CpGs except cg06644428 were 0.46–0.93; Spearman correlation coefficients between cg06644428 and other CpGs were 0.18–0.66 (see Supplemental Material, Table S2).

### Methylation intensities by smoking characteristics

Table 2 shows the association between smoking behavior and methylation intensities at cg05575921 and cg06126421 estimated by linear regression (results for the other seven CpGs, which showed very similar patterns, are presented in Supplemental Material, Table S3). Compared with participants who never smoked, current and former smokers had the lowest and intermediate methylation levels at both CpGs, respectively. Methylation intensities were inversely associated with both current and lifetime smoking intensity, and were positively associated with time since cessation. Estimated dose–response curves for smoking behavior with methylation intensity at the two CpGs are shown in Figure 1. A steep decrease in methylation intensity was observed with increasing

| Characteristic                  | AHRR (cg05575921) | 6p21.33 (cg06126421) |
|--------------------------------|-------------------|----------------------|
|                                | Median (Q1–Q3)    | p-Value              | Median (Q1–Q3)    | p-Value              |
| **Sex**                        |                   |                      |                   |                      |
| Male                           | 500 (50.0)        | 0.82 (0.70–0.87)     | 0.63 (0.57–0.69)  |                      |
| Female                         | 500 (50.0)        | 0.88 (0.84–0.90)     | < 0.0001          | 0.69 (0.65–0.73)     |
| **Age (years)**                |                   |                      |                   |                      |
| < 60                           | 339 (33.9)        | 0.85 (0.74–0.89)     | 0.61 (0.68–0.72)  |                      |
| 60–64                          | 289 (28.9)        | 0.86 (0.77–0.89)     | 0.66 (0.59–0.71)  |                      |
| 65–69                          | 226 (22.6)        | 0.86 (0.79–0.89)     | 0.66 (0.59–0.71)  |                      |
| 70–75                          | 146 (14.6)        | 0.86 (0.79–0.90)     | 0.20 (0.57–0.70)  | 0.20 (0.57–0.70)     |
| **Smoking status**             |                   |                      |                   |                      |
| Never smoker                   | 469 (48.0)        | 0.88 (0.86–0.90)     | 0.70 (0.66–0.73)  |                      |
| Former smoker                  | 323 (33.0)        | 0.83 (0.77–0.87)     | 0.64 (0.58–0.69)  |                      |
| Current smoker                 | 186 (19.0)        | 0.63 (0.56–0.70)     | < 0.0001          | 0.57 (0.51–0.62)     |
| **BMI (kg/m²)**                |                   |                      |                   |                      |
| Underweight (< 18.5)           | 8 (0.8)           | 0.55 (0.66–0.87)     | 0.55 (0.50–0.66)  |                      |
| Normal weight (18.5 to < 25.0) | 243 (24.4)        | 0.86 (0.73–0.89)     | 0.66 (0.59–0.71)  |                      |
| Overweight (25.0 to < 30.0)    | 483 (48.5)        | 0.86 (0.77–0.89)     | 0.67 (0.60–0.71)  |                      |
| Obese (≥ 30.0)                 | 263 (26.4)        | 0.88 (0.78–0.89)     | 0.07 (0.66–0.72)  | 0.12                 |
| **Physical activity**          |                   |                      |                   |                      |
| Inactive                       | 203 (20.3)        | 0.86 (0.74–0.89)     | 0.59 (0.57–0.71)  |                      |
| Insufficient                   | 438 (43.8)        | 0.86 (0.77–0.89)     | 0.66 (0.59–0.71)  |                      |
| Sufficient                     | 358 (35.8)        | 0.86 (0.78–0.89)     | 0.97 (0.67–0.70)  | 0.12                 |
| **Diabetes**                   |                   |                      |                   |                      |
| Not prevalent                  | 837 (83.8)        | 0.86 (0.77–0.89)     | 0.66 (0.59–0.71)  |                      |
| Prevalent                      | 162 (16.2)        | 0.86 (0.78–0.89)     | 0.43 (0.67–0.72)  | 0.07                 |
| **Cardiovascular disease**     |                   |                      |                   |                      |
| Not prevalent                  | 794 (79.4)        | 0.86 (0.78–0.89)     | 0.67 (0.60–0.71)  |                      |
| Prevalent                      | 216 (21.6)        | 0.84 (0.74–0.89)     | 0.08 (0.56–0.69)  | 0.0003               |
| **Cancer**                     |                   |                      |                   |                      |
| Not prevalent                  | 934 (93.4)        | 0.86 (0.78–0.89)     | 0.66 (0.60–0.71)  |                      |
| Prevalent                      | 66 (6.6)          | 0.86 (0.76–0.89)     | 0.71 (0.59–0.71)  | 0.37                 |

Q, quartile.  
*Data for the other seven CpGs are reported in Supplemental Material, Table S1. **Kruskal–Wallis test for group differences.  
*Data missing for 22 participants. *Data missing for 3 participants. *Data missing for 1 participant.  
Categories are defined as follows: inactive, < 1 hr/week of physical activity; sufficient, ≥ 2 hr/week of vigorous physical activity or ≥ 2 hr/week of light physical activity; insufficient, other.
smoking intensity up to approximately 15 cigarettes per day and with increasing cumulative smoking up to approximately 30–40 pack-years, followed by further gradual decrease at higher current and lifetime smoking intensity. Among former smokers, methylation intensity steadily increased with time since cessation up to approximately 20–25 years after quitting and leveled off thereafter. Similar patterns of dose–response curves were also observed for most of the other seven CpGs (with exception of cg05951221, cg23576855, and cg06644428 for current smoking intensity; cg06644428 for pack-years; and cg23576855 and cg06644428 for time after quitting smoking (see Supplemental Material, Figure S1)).

**Methylation intensities and mortality.** Supplemental Material, Figure S2 depicts the survival experience according to quartiles of methylation intensity at the nine CpGs: a gradient of lower survival among participants with lower methylation levels was observed for 7 of the nine CpGs (all except cg23576855 and cg06644428). The associations of methylation intensity at the individual CpGs with all-cause mortality are further presented in Supplemental Material, Table S4. After multivariable adjustment, the strongest and statistically significant associations were estimated for two CpGs (cg05575921 and cg06126421), with HR = 2.45 [95% confidence interval (CI): 1.26, 4.79] and HR = 2.34 (95% CI: 1.27, 4.30), respectively, for the lowest quartile compared with the highest quartile. In addition, a decrease in methylation intensity by one standard deviation was associated with an increase in all-cause mortality by 15%–60% for seven CpGs (all except cg23576855 and cg06644428). In addition, a 1-SD decrease in methylation intensity was associated with higher all-cause mortality for seven CpGs (HR 1.15–1.59, with \( p < 0.05 \) for 5 CpGs); HRs for cg23576855 and cg06644428 were 0.97 and 1.00, respectively.

Table 3 shows the associations of score-based methylation with all-cause and cause-specific mortality. Multivariable-adjusted HRs for cardiovascular, cancer, and other mortality were 7.41 (95% CI: 2.81, 19.54), 2.48 (95% CI: 1.01, 6.08), and 2.78 (95% CI: 0.97, 7.98), respectively, for participants in the lowest quartile of methylation for both cg05575921 and cg06126421 compared with participants who were not in the lowest quartile of methylation for either CpG. By contrast, the strong associations between current smoking and all mortality outcomes were substantially attenuated or disappeared after adjustment for methylation-based score. Joint classification by sex and methylation demonstrated clear dose–response relationships of the methylation score with mortality in both sexes (see Supplemental Material, Table S5).

**Methylation intensity and fatal cardiovascular risk prediction.** Table 4 and Supplemental Material, Table S6 present the increment in the performance indicators of the SCORE in prediction of fatal CVD by adding methylation intensity. The largest improvement was observed when including cg05575921 and cg06126421: Harrell’s C-statistics increased from 0.754 for the SCORE-only model to 0.822 and from 0.730 to 0.779 after correction for over-optimism (Table 4). Adding the two CpGs also resulted in 18 cases and 82 controls moving up and 11 cases and 151 controls moving down, which resulted in a NRI of 21.92% (\( p = 0.049 \)) and a significant IDI of 3.73% (\( p = 0.005 \)). Additionally adding methylation at other CpGs did not lead to a further improvement in the prediction of fatal CVD mortality (see Supplemental Material, Table S6). Even though NRI and IDI increased with additional CpGs included in the model, a substantial proportion of controls, who were supposed to move to lower risk categories, moved to higher risk categories along with cases moving to higher risk categories. The improvement in risk prediction became larger after excluding participants with CVD at baseline (\( n = 216 \); see Supplemental Material, Table S7). The Gronnesby–Borgan test indicated that the new model was also well-calibrated in both full and sensitivity analyses (all \( p > 0.05 \)).

### Table 2. Association between smoking behavior and methylation intensity.*

| Characteristic | AHR(cg05575921) | Reg. coeff. | p-Value | AHR(cg06126421) | Reg. coeff. | p-Value |
|---------------|-----------------|------------|---------|-----------------|------------|---------|
| **Smoking status** | | | | | |
| Never smoker | Reference | | Reference | | |
| Former smoker | Reference | | Reference | | |
| Current smoker | Reference | | Reference | | |
| Current intensity of smoking†(average number of cigarettes/day) | | | | |
| 0 (never and former smokers) | Reference | | Reference | | |
| 10–19 | 0.14 (–0.17, –0.11) | < 0.0001 | 0.20 (–0.22, –0.17) | < 0.0001 |
| 20–29 | 0.22 (–0.23, –0.20) | < 0.0001 | 0.11 (–0.12, –0.09) | < 0.0001 |
| ≥ 30 | 0.27 (–0.31, –0.23) | < 0.0001 | 0.13 (–0.17, –0.10) | < 0.0001 |
| Cumulative dose of smoking (pack-years)‡ | | | | |
| 0 (never smokers) | Reference | | Reference | | |
| < 10 | 0.03 (–0.05, –0.01) | 0.001 | 0.02 (–0.04, –0.01) | 0.003 |
| 10–19 | 0.09 (–0.10, –0.07) | < 0.0001 | 0.06 (–0.07, –0.04) | < 0.0001 |
| 20–29 | 0.12 (–0.13, –0.09) | < 0.0001 | 0.08 (–0.08, –0.06) | < 0.0001 |
| ≥ 30 | 0.19 (–0.21, –0.18) | < 0.0001 | 0.11 (–0.12, –0.10) | < 0.0001 |
| Time since cessation of smoking (years)§ | | | | |
| 0 (current smokers) | Reference | | Reference | | |
| < 2 | 0.02 (–0.02, 0.06) | 0.31 | 0.002 (–0.04, 0.03) | 0.93 |
| 2–4 | 0.11 (0.08, 0.13) | < 0.0001 | 0.04 (0.01, 0.06) | 0.002 |
| 5–9 | 0.13 (0.11, 0.15) | < 0.0001 | 0.03 (0.01, 0.05) | 0.007 |
| 10–20 | 0.17 (0.15, 0.19) | < 0.0001 | 0.07 (0.05, 0.08) | < 0.0001 |
| ≥ 20 | 0.21 (0.19, 0.22) | < 0.0001 | 0.09 (0.07, 0.10) | < 0.0001 |

*Results from linear regression, adjusted for sex, age, BMI (\( < 25 \) kg/m², 25.0 to \( < 30.0 \) kg/m², \( ≥ 30.0 \) kg/m²), physical activity (inactive, insufficient, sufficient), prevalence of cardiovascular disease, diabetes, and cancer, and batch effect. †Data missing for 22 participants. ‡Data missing for 26 participants. ‡Data missing for 88 participants. §Data missing for 1 participant.

**Discussion**

In this population-based cohort study, we found clear dose–response relationships of current and lifetime smoking exposure and time since smoking cessation with site-specific methylation, which were consistent among six CpGs located in *AHRR* (cg05575921, cg21161138), *F2RL3* (cg03636183), *2q37.1* (cg21566642, cg01940273), and *6p21.2* (cg06126421). Methylation at seven CpGs (all above + cg05951221) was also associated with mortality outcomes to various extents. A score based on methylation at the top two CpGs (cg05575921 and cg06126421) provided very strong associations with all-cause, cardiovascular, and cancer mortality. Moreover, integrating methylation at these two CpGs into the conventional risk factors substantially improved the accuracy of predicting fatal cardiovascular risk and reclassified a substantial proportion of individuals to higher or lower risk categories.

A biomarker reflecting long-term past smoking exposure is desirable for accurate evaluation of smoking cessation and for assessment of smoking-related disease risk (CDC 2010). DNA methylation biomarkers might be promising candidates for this purpose. Methylation at nine loci targeted in our study was reported to be strongly associated with smoking exposure by both previous genome-wide methylation studies (Shenker et al. 2013a; Zeilinger et al. 2013). In the present study, distinct and rather consistent dose–response patterns of
methylation with respect to both lifetime cumulative smoking exposure and time since cessation were observed for six of the nine CpGs, which are, of note, similar to the dose–response patterns observed between smoking and smoking-related diseases. For example, cardiovascular risk increases sharply at low levels of cigarette consumption and then plateaus at higher levels of smoking (CDC 2010); the reduction of cardiovascular risk becomes evident within the initial years after quitting smoking and remains slightly elevated for more than a decade (CDC 2010; Kramer et al. 2006; Lightwood and Glantz 1997). The observed dose–response pattern of these six CpGs with current and lifetime smoking behavior was also consistent with dose–response patterns of methylation at the F2RL3 gene previously identified by our group in a large study specifically focusing on this site (Zhang et al. 2014). In addition, in the study by Shenker et al. (2013b), a methylation index combining four of the nine CpGs investigated in our study (cg23576855, cg06644428, cg21566642, and cg06126421) provided superior performance in distinguishing former smokers from never smokers [area under the curve (AUC) = 0.82 (95% CI: 0.96, 0.99)] compared with cotinine [AUC = 0.47 (95% CI: 0.32, 0.63)]. Our present study, in which we addressed associations of methylation patterns with both smoking and smoking-related mortality, suggested that the identified DNA methylation biomarkers might be markers of cumulative smoking exposure-associated risk.

The AHRR gene, known as a tumor repressor (Zudaire et al. 2008), codes a protein involved in multiple physiological pathways, such as metabolism of tobacco smoke components (Kasai et al. 2006; Moennikes et al. 2004) and regulation of cell proliferation and differentiation (Haarman-Stemmann et al. 2007; Pot 2012). Hypomethylation of cg05575921 at AHRR has been reported to be associated with increasing lymphoblast AHRR gene expression in vivo (Monick et al. 2012). It has also been observed that AHRR expression in human lung tissues was inversely correlated with methylation levels of cg23576855 and cg21161138 at AHRR, with 5.7-fold increased expression in five current smokers compared with five nonsmokers (Shenker et al. 2013a). AHRR and the aryl hydrocarbon receptor (AHR) constitute a feedback loop in which the AHR heterodimer activates the expression of the AHRR gene, and the expressed AHRR inhibits the function of AHR in oncogenesis (Mimura et al. 1999). Tobacco smoking has been shown to trigger the production of AHR that mediates dioxin toxicity and other pathological effects (Martey et al. 2005; Meek and Finch 1999). Therefore, it is plausible to assume that demethylation/overexpression of the AHRR gene may result from a smoking-induced increase in AHR activation. The gene product of F2RL3, thrombin protease-activated receptor-4 (PAR-4), plays roles in inflammatory reactions and blood coagulation (Leger et al. 2006), and other pathophysiology commonly described in smoking-induced conditions (Leone 2007; Rahman and Laher 2007). Hypomethylation at F2RL3 has been suggested to be strongly associated with mortality in a cohort of 1,206 patients with stable CVD (Breitling et al. 2012). Interestingly, methylation at four CpGs assessed in our study [AHRR (cg05575921), F2RL3 (cg03636183), 2q37.1 (cg21566642), and 6p21.22 (cg06126421)] were recently found to be associated with a metabolic indicator of complex disorders, 4-vinylphenol sulfate (Petersen et al. 2014). Of note, this metabolic marker has also been reported to be associated with smoking (Manini et al. 2003). Although the potential joint or

Figure 1. Dose–response relationships between smoking behavior and methylation intensity (results from restricted cubic spline regression adjusted for potential confounding factors). CL, confidence limit. (A) Dose–response relationship between current intensity of smoking and methylation intensity at AHRR (cg05575921; left), and 6p21.22 (cg06126421; right); never and former smokers were defined as reference, with current smoking intensity = 0. (B) Dose–response relationship between cumulative dose of smoking and methylation intensity at AHRR (cg05575921; left), and 6p21.22 (cg06126421; right); never smokers were defined as reference, with pack-years = 0. (C) Dose–response relationship between time since cessation of smoking and methylation intensity at AHRR (cg05575921; left), and 6p21.22 (cg06126421; right) among former smokers; current smokers were defined as reference, with time since cessation = 0.
independent epigenetic role of the various loci remains to be clarified, these findings, as well as the disappearance or attenuation of association between smoking and mortality outcomes after adjustment for methylation at these CpGs in the present study, suggest that multiple DNA methylation sites are involved in mediating smoking-related adverse effects.

The much stronger associations of the methylation markers with mortality outcomes, compared with those of commonly studied molecular and genetic biomarkers, and the attenuation or disappearance of the association between current smoking and mortality after adjustment for the methylation markers observed in our study suggest that DNA methylation biomarkers may more accurately summarize individuals’ smoking-related risks that accumulated through past and current exposure, and thus be more informative in risk assessment than self-reported smoking history. To our knowledge, this is the first study to evaluate the improvement in risk assessment of fatal CVD

### Table 3. Methylation score and smoking in relation to mortality outcomes.

| Outcome/methylation score^/ smoking status | n total | Cases | PY | IR^ | HR (95% CI) |
|-------------------------------------------|--------|-------|----|-----|-------------|
| All-cause mortality                        |        |       |    |     |             |
| 0                                         | 677    | 60    | 6716.06 | 0.89 | Reference |
| 1                                         | 151    | 31    | 1431.15 | 2.17 | Reference |
| 2                                         | 172    | 52    | 1546.03 | 3.36 | Reference |
| Never smoker                               | 469    | 45    | 4651.73 | 0.97 | Reference |
| Former smoker                              | 323    | 58    | 3059.15 | 1.90 | Reference |
| Current smoker                             | 186    | 37    | 1766.04 | 2.10 | Reference |
| CVD mortality                              |        |       |    |     |             |
| 0                                         | 677    | 16    | 6680.70 | 0.24 | Reference |
| 1                                         | 151    | 14    | 1431.15 | 0.98 | Reference |
| 2                                         | 172    | 20    | 1525.56 | 1.31 | Reference |
| Never smoker                               | 469    | 17    | 4627.45 | 0.37 | Reference |
| Former smoker                              | 323    | 23    | 3058.06 | 0.75 | Reference |
| Current smoker                             | 186    | 10    | 1745.58 | 0.57 | Reference |
| Cancer mortality                           |        |       |    |     |             |
| 0                                         | 677    | 24    | 6680.70 | 0.36 | Reference |
| 1                                         | 151    | 19    | 1431.15 | 0.98 | Reference |
| 2                                         | 172    | 16    | 1525.56 | 1.05 | Reference |
| Never smoker                               | 469    | 14    | 4627.45 | 0.30 | Reference |
| Former smoker                              | 323    | 21    | 3058.06 | 0.69 | Reference |
| Current smoker                             | 186    | 13    | 1745.58 | 0.74 | Reference |
| Other mortality                            |        |       |    |     |             |
| 0                                         | 677    | 17    | 6680.70 | 0.22 | Reference |
| 1                                         | 151    | 16    | 1431.15 | 0.98 | Reference |
| 2                                         | 172    | 11    | 1525.56 | 0.85 | Reference |
| Never smoker                               | 469    | 15    | 4627.45 | 0.22 | Reference |
| Former smoker                              | 323    | 8     | 3058.06 | 0.43 | Reference |
| Current smoker                             | 186    | 13    | 1745.58 | 0.63 | Reference |

Abbreviations: HR, hazard ratio; IR, incidence rate; PY, person-years.

### Table 4. Evaluation of the SCORE and methylation intensity in prediction of fatal CVD (controlling for batch effect).

| Characteristic | SCORE | SCORE + cg0557921 | SCORE + cg06126421 | SCORE + cg0557921 + cg06126421 |
|----------------|-------|------------------|--------------------|----------------------------------|
| Overall model fit | -2 LOG L | df; p-value | 623.93; 5; < 0.0001 | 601.82; 10; < 0.0001 | 602.21; 10; < 0.0001 | 597.54; 11; < 0.0001 |
| AIC | 633.93 | 621.82 | 622.52 | 619.54 |
| LR test p-value^2 | 0.0005 | 0.0005 | 0.0005 | 0.0002 |
| Harrell’s C-statistics (95% CI) | 0.754 (0.691, 0.818) | 0.810 (0.752, 0.867) | 0.806 (0.748, 0.864) | 0.822 (0.765, 0.879) |
| Optimum-corrected Harrell’s C-statistics (95% CI) | 0.736 (0.676, 0.791) | 0.773 (0.687, 0.832) | 0.768 (0.678, 0.830) | 0.779 (0.693, 0.840) |

Abbreviations: AIC, Akaike information criterion; IDI, integrated discrimination improvement; LOG L, log-likelihood; LR, likelihood ratio; n MODEL, number of expected events; n total, number of observed events; NRI, net reclassification improvement; SCORE, Systematic Coronary Risk Evaluation chart (age, sex, systolic blood pressure, current smoking, and total cholesterol).

Comparison of SCORE + methylation model with SCORE model by likelihood ratio test.
when adding DNA methylation biomarkers to conventional risk factors. The increment in C-statistics by adding the methylation intensity at cg05575921 and cg06126421 (approximately 0.04) was much larger than the increment seen by adding a multimarker score in the Framingham Heart Study (C-statistics for model of major cardiovascular events increased by 0.01) (Wang et al. 2006). In another large population-based cohort, the investigators evaluated six novel biomarkers for cardiovascular risk prediction along with the conventional markers and reported the NRI was 0.00% and 4.70% for cardiovascular events and coronary events, respectively (Melander et al. 2009). They obtained improved NRI by restricting the analyses to individuals with intermediate risk; the reclassification, however, was essentially confined to down-classification of participants without events. Of note, the proportion of reclassified participants was substantial in our study, and consisted of not only down-classification of individuals without events but also up-classification of individuals with events. Given that nearly 22% of participants were reclassified, inclusion of smoking-associated methylation markers into the routine screening programs, as such the SCORE risk estimation system, would benefit a substantial proportion of individuals in the population setting and could greatly promote cost effectiveness of CVD prevention and therapy. On the other hand, our study was an exploratory investigation on CVD risk prediction using methylation markers based on a limited number of total cardiovascular deaths, thus our findings need to be validated in an independent population. The performance of these methylation markers for predicting risk of nonfatal or subtype of fatal CVD, such as coronary and non-coronary heart disease, needs to be evaluated in further studies with high-quality assessment of CVD risk factors as well as CVD events. In addition, to examine the generalizability of the current finding, the performance of methylation markers should also be assessed in relation to other well-established risk scores, such as the Framingham score, and in geographically different populations.

Our study has specific strengths and limitations. Strengths of our study are the population-based prospective study design with comprehensive information on smoking exposure and a variety of covariates, as well as long-term complete mortality follow-up data. A limitation is that the limited numbers of cause-specific deaths prevent the analyses from going into more detail, such as sex-specific examination of CVD risk prediction or investigation of deaths from well-known smoking-associated subtypes of cancer (CDC 2014; Ezzati et al. 2005a). Future studies with large numbers of participants would be desirable to further validate our findings. Information on cause of death was based on death certificates, which are known to be less than perfect. However, potential misclassification between the broad categories of causes of deaths assessed in our study is likely to be much less relevant than potential misclassification between specific causes; given the rather consistent findings of an inverse association with methylation intensity for all categories of causes of deaths, such misclassification might have had only a small impact on the observed results. An additional limitation of our study is that methylation was measured from whole blood, without possibilities for differentiating DNA methylation between various cell types. It might therefore be conceivable that differences in methylation might, in part, reflect different distribution of leukocyte cell types. However, even if the difference in methylation we observed was primarily or partly due to shifts in leukocyte distribution, their use as biomarkers for characterizing smoking exposure or risk prediction would not have been invalidated. On the contrary, given that DNA from whole blood is more readily obtainable in most clinical and epidemiological settings, biomarkers based on whole blood may be more relevant for clinical practice. Finally, our results are based on a single study and might be overoptimistic because only the CpG sites that performed best in the exploratory phase of the study were used to create the model and outcome classification. Further validation in independent studies should therefore be the aim for future studies.

Despite its limitations, our study strongly supports the potential utility of DNA methylation markers as indicators for both current and lifetime smoking exposure and for predicting mortality outcomes, in particular for cardiovascular mortality. Incorporation of methylation biomarkers into conventional risk factors might be a promising approach to improve cardiovascular risk assessment and disease prevention, which needs to be further validated and confirmed in additional studies with a large number of participants and detailed assessment of known determinants of CVD.

REFERENCES

Baner A, Zucknick M, Hielscher T, Ittrich C, Mansmann U. 2010. High-dimensional Cox models: the choice of penalty as part of the model building process. Biom J 52:90–99.
Brettle RP, Salzmann K, Rothenbacher D, Burwinkel B, Brenner H. 2012. Smoking, F2RL3 methylation, and prognosis in stable coronary heart disease. Eur Heart J 33:2841–2848.
CDC (Centers for Disease Control and Prevention). 2010. How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta, GA:CDC. Available: http://www.cdc.gov/nchlà/reportsoncancer/ncbi.nlm.nih.gov/books/NBK53017/ [accessed 11 May 2015].
CDC. 2014. The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General. Atlanta, GA:CDC. Available: http://www.cdc.gov/nchà/reportsoncancer/ncbi.nlm.nih.gov/books/NBK179276/ [accessed 11 May 2015].
Connor Gorber S, Schofield-Hurwitz S, Hardt J, Levassuer G, Tremblay M. 2009. The accuracy of self-reported smoking: a systematic review of the relationship between self-reported and cotinine-assessed smoking status. Nicotine Tob Res 11:12–24.
Conroy RM, Pyörälä K, Fitzgerald AP, Sains S, Menotti A, De Backer G, et al. 2003. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. Eur Heart J 24:987–1003.
Cook NR. 2007. Use and misuse of the receiver operating characteristic curve in risk prediction. Circulation 115:928–935.
Cook NR. 2010. Methods for evaluating novel biomarkers—a new paradigm. Int J Clin Pract 64:1723–1727.
Decramer M, Janssens W, Miravitlles M. 2012. Chronic obstructive pulmonary disease. Lancet 379:1341–1351.
Desquieu L, Mariotti F. 2010. Dose-response analyses using restricted cubic spline functions in public health research. Stat Med 29:1037–1057.
Ezzati M, Henley SJ, Lopez AD, Thun MJ. 2005a. Role of smoking in global and regional cancer epidemiology: current patterns and data needs. Int J Cancer 116:963–971.
Ezzati M, Henley SJ, Thun MJ, Lopez AD. 2005b. Role of smoking in global and regional cardiovascular mortality. Circulation 112:488–497.
Goeman JJ. 2010. Li penalized estimation in the Cox proportional hazards model. Biom J 52:78–94.
Goeman JJ, Meijer R, Chaturvedi N. 2014. Package ‘penalized’. Available: https://cran.r-project.org/web/packages/penalized/index.html.
Haarmann-Stemmann T, Bothe H, Kohli A, Sydlik U, Abel J, Frutsche E. 2007. Analysis of the transcriptional regulation and molecular function of the aryl hydrocarbon receptor repressor in human cell lines. Drug Metab Dispos 35:2262–2269.
Harrel FE Jr, Lee KL, Mark DB. 1996. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med 15:361–387.
Joubert BR, Häger SE, Nilsson IM, Wang X, Volland SE, Murphy SK, et al. 2012. 450K array-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. Environ Health Perspect 120:1425–1431; doi:10.1289/ ehp.1205412.
Kasai A, Hiramatsu N, Hayakawa K, Yao J, Maeda S, Kitamura M. 2008. High levels of dioxin-like potential in cigarette smoke evidenced by in vitro and in vivo biosensing. Cancer Res 68:7143–7150.
Kramer A, Jansen AC, van Aalst-Cohen ES, Tanck MW, Kastelein JJ, Zwinderman AH. 2006. Relative risk for cardiovascular atherosclerotic events after smoking cessation: 6–8 years excess risk in individuals with familial hypercholesterolemia. BMC Public Health 6:262; doi:10.1186/1471-2458-6-262.
Leger AJ, Covic L, Kuliopulos A. 2006. Protease-activated receptors in cardiovascular diseases. Circulation 114:1070–1077.
Leone A. 2007. Smoking, haemostatic factors, and cardiovascular risk. Curr Pharm Des 13:1661–1667.
Lightwood JM, Glantz SA. 1997. Short-term economic biosensing. Cancer Res 66:7143–7150.
Luo Y, Liu Y, Cai Y, Zhang Z, Zhao X, Wang X, et al. 2015. Abnormal DNA methylation in newborns related to maternal smoking during pregnancy. Environ Health Perspect 120:1425–1431; doi:10.1289/ ehp.1205412.
Lin DY, Wei LJ, Ying ZL. 1993. Checking the Cox model with cumulative sums of martingale-based residuals. Biometrika 80:557–572.
Mansmann U. 2010. High-dimensional Cox models: the choice of penalty as part of the model building process. Biom J 52:90–99.
Mansmann U. 2010. High-dimensional Cox models: the choice of penalty as part of the model building process. Biom J 52:90–99.
Mansmann U. 2010. High-dimensional Cox models: the choice of penalty as part of the model building process. Biom J 52:90–99.
Mansmann U. 2010. High-dimensional Cox models: the choice of penalty as part of the model building process. Biom J 52:90–99.
Zhang et al.

Lodovici M, Bigagli E. 2009. Biomarkers of induced active and passive smoking damage. Int J Environ Res Public Health 6:874–888.

Löw M, Stegmaier C, Ziegler H, Rothenbacher D, Brenner H, ESTHER study. 2004. Epidemiological investigations of the chances of preventing, recognizing early and optimally treating chronic diseases in an elderly population (ESTHER study) [in German]. Dtsch Med Wochenschr 129:2643–2647.

Manini P, De Palma G, Andreoli R, Goldoni M, Poli D, Lasagni G, et al. 2003. Urinary excretion of 4-vinyl phenol after experimental and occupational exposure to styrene [in Italian]. G Ital Med Lav Ergon 25(suppl 3):61–62.

Martey CA, Baglole CJ, Gasiewicz TA, Sime PJ, Phipps RP. 2005. The aryl hydrocarbon receptor is a regulator of cigarette smoke induction of the cyclooxygenase and prostaglandin pathways in human lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 289:L391–L399.

May S, Hasmr DW. 2004. A cautionary note on the use of the Grønnesby and Borgan goodness-of-fit test for the Cox proportional hazards model. Lifetime Data Anal 10:283–291.

Meek MD, Finch GL. 1999. Diluted mainstream cigarette smoke condensates activate estrogen receptor and aryl hydrocarbon receptor-mediated gene transcription. Environ Res 80:9–17.

Melander O, Newton-Cheh C, Almgren P, Hedblad B, Berglund G, Engström G, et al. 2009. Novel and conventional biomarkers for prediction of incident cardiovascular events in the community. JAMA 302:49–57.

Miao Y, Cenzer IS, Kirby KA, Boscardin WJ. 2013. Estimating Harrell’s optimism on predictive indices using bootstrap samples. Paper 504-2013. In: Proceedings of the SAS® Global Forum 2013 Conference. Cary, NC:SAS Institute Inc. Available: http://support.sas.com/resources/papers/proceedings13/504-2013.pdf [accessed 11 May 2015].

Miller SA, Dyske DD, Polaseky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215; doi:10.1093/nar/16.3.1215.

Mimura J, Ema M, Sugawa K, Fujii-Kuriyama Y. 1999. Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. Genes Dev 13:20–25.

Moennikes K, Loepen S, Buchmann A, Andersson P, Ittrich C, Poellinger L, et al. 2004. A constitutively active dioxin/aryl hydrocarbon receptor promotes hepatocarcinogenesis in mice. Cancer Res 64:4707–4710.

Monick MM, Beach SR, Plume J, Sears R, Gerrard M, Brody GH, et al. 2012. Coordinated changes in AhRR methylation in lymphoblasts and pulmonary macrophages from smokers. Am J Med Genet B Neuropsychiatr Genet 159B:141–151.

Pencina MJ, D’Agostino RB Sr, D’Agostino RB Jr, Vasan RS. 2008. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med 27:157–172.

Petersen AK, Zeilinger S, Kühnel B, Klopp N, Baurecht H, Kleinschmidt A, Gieger C, et al. 2014. Epigenetics meets metabolomics: an epigenome-wide association study with blood serum metabolic traits. Hum Mol Genet 23:534–546.

Peterson AK, Zeilinger S, Kastenmüller G, Römisch-Margl W, Brugger M, Peters A, et al. 2014. Epigenetics meets metabolomics: an epigenome-wide association study with blood serum metabolic traits. Hum Mol Genet 23:534–546.

Pot C. 2012. Aryl hydrocarbon receptor controls regulatory CD4+ T cell function. Swiss Med Wkly 142:w13592; doi:10.4414/smw.2012.13592.

Rahman MM, Lafer I. 2007. Structural and functional alteration of blood vessels caused by cigarette smoke: an overview of molecular mechanisms. Curr Vasc Pharmacol 5:276–292.

Rebagliato M. 2002. Validation of self reported gene in an elderly population (ESTHER study) [in Italian]. Dtsch Med Wochenschr 129:2643–2647.

Shi Y, Yang R, Breitling LP, Brenner H. 2006. Multiple biomarkers for the prediction of first major cardiovascular events and death. N Engl J Med 355:2631–2639.

Zeilinger S, Kühnel B, Klopp N, Baurecht H, Kleinschmidt A, Gieger C, et al. 2013. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. PLoS One 8:e63812; doi:10.1371/journal.pone.0063812.

Zhang Y, Yang R, Burwinkel B, Breitling LP, Brenner H. 2014. F2RL3 methylation as a biomarker of current and lifetime smoking exposures. Environ Health Perspect 122:131–137; doi:10.1289/ehp.1306937.

Zudaire E, Cuesta N, Murty V, Woodson K, Adams L, Miller SA, Dykes DD, Polaseky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215; doi:10.1093/nar/16.3.1215.