Hypomethylation of smoking-related genes is associated with future lung cancer in four prospective cohorts

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DNA hypomethylation in certain genes is associated with tobacco exposure but it is unknown whether these methylation changes translate into increased lung cancer risk. In an epigenome-wide study of DNA from pre-diagnostic blood samples from 132 case–control pairs in the NOWAC cohort, we observe that the most significant associations with lung cancer risk are for cg05575921 in \textit{AHRR} (OR for 1 s.d. = 0.37, 95% CI: 0.31–0.54, \textit{P}-value = 3.3 \times 10^{-11}) and cg03636183 in \textit{F2RL3} (OR for 1 s.d. = 0.40, 95% CI: 0.31–0.56, \textit{P}-value = 3.9 \times 10^{-10}), previously shown to be strongly hypomethylated in smokers. These associations remain significant after adjustment for smoking and are confirmed in additional 664 case–control pairs tightly matched for smoking from the MCCS, NSHDs and EPIC HD cohorts. The replication and mediation analyses suggest that residual confounding is unlikely to explain the observed associations and that hypomethylation of these CpG sites may mediate the effect of tobacco on lung cancer risk.

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NA methylation has recently emerged as an important marker of current and past smoking habits. Smoking is a leading cause of death worldwide and has been identified as a major risk factor for several diseases including cancer, cardiovascular and respiratory diseases. The carcinogenic effect of tobacco smoking persists for decades after smoking cessation, and former smokers remain at increased risk of lung cancer for 20 years or longer.

Using an epigenome-wide methylation study approach, we previously demonstrated that tobacco smoking alters DNA methylation patterns, particularly in CpG sites of the AHRR and F2RL3 genes. These results have been extensively replicated by other studies. In particular, our previous study of 1,000 cancer, cardiovascular and respiratory diseases. The identification as a major risk factor for several diseases including lung cancer for 20 years or longer.

Smoking cessation, and former smokers remain at increased risk of lung cancer for 20 years or longer. The odds ratio for lung cancer was 7.38 for former and current smokers grouped together (95% confidence interval (CI) 3.99–16.66), 6.16 (95% CI: 2.65–15.13) for former smokers and 10.13 (95% CI: 4.56–24.23) for current smokers.

Table 1 shows the top-ranked CpG sites for the locus-by-locus epigenome-wide risk analysis, and includes all CpG sites with Bonferroni-corrected P-values below 0.05. All top-ranked CpGs showed inverse associations with risk, indicating hypomethylation in cancer cases. Supplementary Table 1 shows the main information about involvement in cancer pathways for the probes listed in Table 1: for all the CpGs except two (cg02451831 and cg03898802) there is evidence of involvement in cancer pathways. CpGs in the AHRR and F2RL3 genes displayed the most significant associations with risk consistent with previous observations of smoking being associated with reduced methylation in healthy subjects. In the following analyses, we exclusively focus on these two genes from Table 1, because they are the only ones strongly associated with smoking. In particular, the cg05575921 probe in the AHRR gene emerged as the CpG site most strongly associated with both tobacco exposure and lung cancer risk (odds ratio (OR) for lung cancer per 1 standard deviation (s.d.) of beta: 0.37, 95% CI: 0.31–0.54, P-value = 3.33 × 10−11). Sensitivity analyses excluding cases with time from blood collection to diagnosis of <2 years showed no significant differences in effect estimates (OR: 0.36, 95% CI: 0.27–0.52 for cg05575921 and OR: 0.40, 95% CI: 0.29–0.56 for cg03636183). Supplementary Table 2 shows the results of the analyses stratified by time to diagnosis (less and more than 5 years). Associations were slightly stronger for <5 years to diagnosis but these were unlikely to reflect reverse causation as they were also evident for more than 5 years to diagnosis.

Table 2 shows the results for the probes associated with cancer risk in the AHRR and F2RL3 genes after adjustment for smoking (for example, smoking status coded as never, former, current): the overall association remained basically unchanged (OR for 1 s.d. = 0.39, 95% CI: 0.24–0.61, P-value = 2.55 × 10−5 for cg05575921 and OR for 1 s.d. = 0.51, 95% CI: 0.35–0.73, P-value = 4.19 × 10−4 for cg03636183).

Validation sets. To validate our results arising from the NOWAC study, we analysed the cg05575921 and cg03636183 probes in three independent samples: a case–control study nested within MCCS including 367 case–control pairs, a case–control study nested within MCCS including 367 case–control pairs, a case–control study nested within MCCS including 367 case–control pairs, a case–control study nested within MCCS including 367 case–control pairs.

Table 1 | Top-ranked CpG sites for the locus-by-locus risk analysis in NOWAC data (discovery set): CpGs in the AHRR and F2RL3 genes display the most significant inverse associations with risk (hypomethylation in cases).

| Probe name | Gene name | Chromosome | Position | Region | OR for 1 s.d. | 95% CI | P-value | P-value Bonferroni |
|------------|-----------|-------------|----------|--------|---------------|--------|---------|------------------|
| cg05575921 | AHRR      | 5           | 373378   | N_Shore| 0.37          | 0.31–0.54 | 3.33 × 10−11 | 1.36 × 10−3 |
| cg03636183 | F2RL3     | 19          | 17000585 | N_Shore| 0.40          | 0.31–0.56 | 3.86 × 10−10 | 1.58 × 10−4 |
| cg21566642 | AHRR      | 2           | 23328329 | Island | 0.36          | 0.23–0.48 | 3.33 × 10−9  | 5.43 × 10−4 |
| cg06126421 | F2RL3     | 6           | 233284934| Island | 0.41          | 0.25–0.49 | 1.52 × 10−9  | 6.21 × 10−4 |
| cg25305703 | CASC21    | 8           | 233284402|        | 0.45          | 0.35–0.60 | 3.38 × 10−8  | 1.34 × 10−2 |
| cg2116138  | AHRR      | 5           | 399360   |        | 0.46          | 0.36–0.62 | 5.01 × 10−8  | 2.04 × 10−2 |
| cg01940273 | F2RL3     | 2           | 26578098 | Island | 0.44          | 0.33–0.60 | 5.21 × 10−8  | 2.32 × 10−2 |
| cg02451831 | KIAA0087  | 7           | 30720080 |        | 0.43          | 0.29–0.57 | 6.55 × 10−8  | 2.67 × 10−2 |
| cg05951221 | BOLA2     | 2           | 233284661| Island | 0.41          | 0.30–0.58 | 8.59 × 10−8  | 3.51 × 10−2 |
| cg04884171 | DOPEY2    | 16          | 128378218| S_Shelf | 0.33          | 0.15–0.41 | 1.18 × 10−8  | 4.82 × 10−2 |
| cg03898802 | AHRR      | 21          | 37617652 | Island | 0.37          | 0.29–0.57 | 1.20 × 10−7  | 4.90 × 10−2 |

CI, confidence interval; OR, odds ratio.

Unconditional logistic regression models were used with DNA methylation levels included as an independent variable and were adjusted for matching variables, micro-array, position of the sample on the micro-array and blood cell composition differentials.
nested within the NSHDS including 234 case–control pairs and a case–control study nested within the EPIC HD cohort, including 63 case–control pairs, all of which were matched on smoking status (see Methods for details).

Consistent with the results from the NOWAC study, methylation levels in the MCCS, NSHDS and EPIC HD studies were clearly inversely associated with lung cancer risk for both the cg05575921 and cg03636183 CpG sites. The overall OR estimates were slightly weaker in MCCS than in NOWAC (OR: 0.62, 95% CI: 0.50–0.78) and OR: 0.61, 95% CI: 0.47–0.79 for cg03636183 in MCCS; OR: 0.45, 95% CI: 0.22–0.92 for cg05575921 and OR: 0.62, 95% CI: 0.38–1.04 for cg0636183 in EPIC HD; Table 2). We note that some attenuation of the overall NOWAC OR estimates is expected as MCCS, NSHDS and EPIC HD studies were matched by smoking status.

Risk prediction model for lung cancer. We applied to the NOWAC cohort a prediction model including smoking status (coded as never, former, current) and methylation as a covariate. This was not feasible for the other cohorts because of matching by smoking. The area under the curve (AUC) of the model increased from 0.71 to 0.76 when adding AHRR-methylation and F2RL3-methylation as categorical variables (above or below the median) and to 0.78 when adding the two as continuous variables.

Lung cancer risk by categories of smoking exposure. To further evaluate the associations of the cg05575921 and cg03636183 CpG sites with lung cancer risk, we conducted stratified risk analysis by categories of smoking status. We found little support for an association between methylation and lung cancer risk, but more consistent with the results from the NOWAC study, methylation was clearly inversely associated with lung cancer risk for both the cg05575921 and cg03636183 CpG sites (Table 2).

Methylation of AHRR and F2RL3 genes in former smokers. The associations between smoking cessation and the mean methylation levels in the cg05575921 probe (AHRR gene) and the cg03636183 probe (F2RL3 gene) in NOWAC are shown in Fig. 1. After smoking cessation, methylation levels increase and after 10
years since quitting appear to approach those of never smokers. This is consistent with the well-documented observation that the risk of lung cancer decreases substantially after smoking cessation.

The effect of smoking (never versus former versus current; time since quitting smoking; smoking duration) on methylation beta levels for cg05575921 and cg03636183 in MCCS and in NSHDS are shown in Fig. 2. Similar to what we observed in NOWAC (Fig. 1), in MCCS and NSHDS, methylation levels in current smokers were lower than methylation levels in never smokers and in former smokers the levels approached those of never smokers with increasing time since cessation.

Comparison of the study groups. Supplementary Table 3 shows a summary of the key characteristics of the study groups. The limitation to a single gender in NOWAC prevented us from making straightforward comparisons between the estimated associations and from investigating differences in lung cancer risk between genders. On the other hand, matching by smoking in MCCS, NSHDS and EPIC HD did not allow us (i) to investigate further the role of methylation as a mediator of the association between smoking and cancer in these cohorts and (ii) to test interactions between smoking variables such as duration or dose.

A future goal will be to repeat the analysis in unrestricted population cohorts.

**Correlation between methylation and expression.** We investigated the correlation between methylation and expression of the two relevant probes using two different sources of data: TCGA (http://cancergenome.nih.gov/) and HapMap (http://hapmap.ncbi.nlm.nih.gov/). In TCGA, we focused on expression (RNA-Seq experiments) and methylation (Illumina HumanMethylation450 BeadChip) of samples of normal tissue (i) from 21 lung adenocarcinoma cases (LUAD—21 methylation-expression pairs) and (ii) from 8 lung squamous cell carcinoma cases (LUSC—8 methylation-expression pairs). *AHRR*-probe methylation seems to be significantly inversely correlated with *AHRR* expression in LUAD and the same trend was found in LUSC (Pearson’s correlation coefficient $= -0.66$, *P* value $<0.01$ in LUAD; *Pearson’s correlation coefficient $= -0.43$, *P* value $= 0.29$ in LUSC). *F2RL3*-probe methylation did not show a statistically significant methylation-expression correlation. Regarding HapMap, we focused on expression (RNA-Seq experiments) and methylation (Illumina HumanMethylation27 BeadChip) data from lymphoblastoid cell lines of 69 HapMap Yoruba individuals. In this case, only the *F2RL3*-probe is present on the platform and its methylation seems to be significantly inversely correlated with *F2RL3* expression (Pearson’s correlation coefficient $= -0.28$, *P* value $<0.01$).

**Mediation analysis.** Although the results described above from the analysis of a discovery set and three validation sets seem to provide evidence that hypomethylation of the cg05575921 and cg03636183 probes is associated with both tobacco exposure and lung cancer risk, the key question is whether their hypomethylation is involved in the causal pathway, or whether they are simply epiphenomena of smoking habits (that is, the association of DNA methylation with lung cancer risk is confounded by smoking). To bring some clarity to this question, we used mediation analysis to quantify the amount by which cg05575921 (*AHRR* gene) and cg03636183 (*F2RL3* gene) methylation might mediate the effect of smoking on lung cancer incidence. This was performed for the NOWAC study as such an analysis was not possible for the MCCS, NSHDS or EPIC HD because of matching by smoking status.
We detected statistically significant results for both components of mediation analysis, the natural direct effect (NDE) of smoking on lung cancer (that is, not mediated) and the natural indirect effect (NIE, that is, the effect mediated by the methylated probe(s)), the two together making up the total causal effect (TCE; see Methods and Table 3, where the underlying identifying assumptions are also stated). The proportion of the smoking-induced risk increase explained by cg05575921 AHRR-probe was found to be $31\%$ (0.31, 95% CI: 0.18–0.46) and $32\%$ (0.32, 95% CI: 0.20–0.53) for the cg03636183 F2RL3-probe. Considering the two genes together, their methylation appeared to mediate $37\%$ (0.37, 95% CI: 0.19–0.66) of the total effect of smoking on lung cancer odds (Fig. 3 and Table 3). The results of mediation analysis were similar when we included the mean methylation of a group of ten AHRR (cg05575921, cg03991871, cg12806681, cg23916896, cg01899089, cg26703534, cg14817490, cg25648203, cg21161138 and cg24090911) and two F2RL3 probes (cg03636183 and cg04259305) located in the body of the gene and significantly associated with lung cancer after false discovery rate correction (data not shown). In conclusion, this analysis suggests (i) that methylation of the smoking-related AHRR and F2RL3 CpG sites might be relevant to lung cancer aetiology and (ii) would explain approximately one-third of the risk increase induced by tobacco exposure.

### Discussion

Tobacco smoking is one of the most important carcinogenic exposures, and continuing smokers experience up to 25% lifetime risk of developing a smoking-related cancer—particularly lung cancer—but the underlying mechanisms by which tobacco carcinogens act on lung cells have been elusive. Mutations, cell proliferation and selection have been hypothesized as complementary mechanisms. Epigenetics has recently emerged as a promising field to illuminate carcinogenetic mechanisms and we have previously shown that smoking is associated with hypomethylation in CpGs of key genes. Here, we present data from four prospective cohort studies that convincingly demonstrate that hypomethylation in specific CpG sites of the AHRR and F2RL3 genes is associated with increased risk of subsequent lung cancer. Although we detected 11 CpG sites in the discovery set that were associated with lung cancer, we selected the AHRR and F2RL3 genes because of their strong

### Table 3 | Mediation analysis of the NOWAC cohort based on g-formula.

| Protein-Probe          | Log OR | s.e.  | P-value | 95% CI       |
|------------------------|--------|-------|---------|--------------|
| **AHRR-cg05575921**    |        |       |         |              |
| TCE                    | 1.83   | 0.29  | <0.001  | (1.37–2.64)  |
| NDE                    | 1.26   | 0.31  | <0.001  | (0.75–2.08)  |
| NIE                    | 0.56   | 0.08  | <0.001  | (0.39–0.73)  |
| Effect mediated        | 0.31   | 0.08  | <0.001  | (0.18–0.46)  |
| **F2RL3-cg03636183**   |        |       |         |              |
| TCE                    | 1.82   | 0.30  | <0.001  | (1.29–2.48)  |
| NDE                    | 1.23   | 0.33  | <0.001  | (0.63–1.93)  |
| NIE                    | 0.59   | 0.09  | <0.001  | (0.43–0.80)  |
| Effect mediated        | 0.32   | 0.08  | <0.001  | (0.20–0.53)  |
| **AHRR-cg05575921 and F2RL3-cg03636183** | | | | |
| TCE                    | 1.79   | 0.30  | <0.001  | (1.28–2.53)  |
| NDE                    | 1.13   | 0.34  | 0.001   | (0.49–1.86)  |
| NIE                    | 0.66   | 0.15  | <0.001  | (0.42–1.09)  |
| Effect mediated        | 0.37   | 0.11  | 0.001   | (0.19–0.66)  |

CI, confidence interval; NDE, natural direct effect; NIE, natural indirect effect; OR, odds ratio; TCE, total causal effect.

TCE, NDE and NIE for the cg05575921 probe in AHRR, for the cg03636183 probe in F2RL3 and for the two probes combined: 31% of the total effect of smoking on lung cancer risk is mediated by AHRR site-specific methylation, 32% of the total effect of smoking on lung cancer risk is mediated by F2RL3 site-specific methylation and 37% of the total effect of smoking on lung cancer risk is mediated by the combined contribution of AHRR and F2RL3 methylation (separate pathways for the two probes).

### Figure 3 | Mediation analysis: graphical representation.

In model A, the percentage of the effect mediated by AHRR-cg05575921 is $31\%$ of the total effect of smoking on lung cancer risk, whereas in model B, the percentage mediated by F2RL3-cg03636183 is $32\%$. The joint mediation effect of these two CpGs is $37\%$ if the two mediators are included together in the model with separate pathways (model C).
Hypomethylation persists in some CpG sites for much longer than the average half-life of circulating white-blood cells, suggesting that stem cells (in the bone marrow in the case of white blood cells, and hypothetically also in the lung) may preserve a ‘memory’ of past exposures in the form of a greater proportion of unmethylated CpG sites versus methylated CpG sites. We speculate that exposure to toxic agents leads to clonal expansion of cells that are hypomethylated in CpGs of genes involved in activation of a pathway reactive to environmental insults, and this imbalance in the proportion of methylated DNA in stem cells persists, remaining mitotically stable through subsequent cell divisions.

The association of hypomethylation at the two selected CpG sites with lung cancer was nominally stronger for former than for current smokers in all our studies but this observation could be due to chance or residual confounding by factors related or unrelated to smoking.

In conclusion, our study shows that smoking-induced hypomethylation in the AHRR and F2RL3 genes is associated with an important risk increase of subsequent lung cancer, and indicates that these specific methylation alterations may mediate the carcinogenic effect of tobacco exposure in lung cancer aetiology.

Methods

Discovery set. Lung cancer cases and matched controls were identified within the Norwegian NOWAC longitudinal cohort. The biobank of the NOWAC cohort was collected in the years 2003–2006. Random samples of Norwegian women were mailed a letter of information with an invitation to receive equipment for blood sampling at the local doctor or other institutions. Those who filled in the eight-page questionnaire and accepted the invitation to donate blood received some months later equipment for blood drawing together with a two-page questionnaire with information on date, lifestyle factors and so on. Around 30,000 women returned by over-night mail two tubes of blood to the Institute of Community Medicine at UiT–The Artic University of Norway. Upon arrival, the citrate glass tube was centrifuged and buffy-coat and plasma frozen immediately at −80°C together with a PAXgene tube. All participants gave informed consent. The study was approved by the Regional Committee for Medical and Health Research Ethics in North Norway. Data storage and linkage to the National Cancer Registry of Norway were approved by the Norwegian Data Inspectorate; follow-up identified 132 eligible cases of lung cancer by 2011. For each case, one control with adequate blood samples was selected matched on time since blood sampling and year of birth in order to control for effects of stressors such as dioxins and polycyclic aromatic hydrocarbons (that are contained in tobacco smoke). We have previously investigated the lung tissue of smokers and non-smokers: methylation levels in the AHRR gene probes were significantly lower (P < 0.001) with a concurrent increase in AHRR expression (P = 0.005) in the lung tissue of current smokers compared with non-smokers. This was further validated in a mouse model of smoke exposure.

F2RL3 is also a functionally relevant gene. It encodes the protease-activated receptor-4, which has been suggested to be involved in the pathophysiology of both cardiovascular and neoplastic diseases. A recent study reported that hypomethylation of F2RL3 is predictive of total mortality and the authors suggested that the adverse health effects of smoking might be mediated in part by pathways related to F2RL3 methylation.

The main question arising from our previous studies of healthy subjects was whether methylation changes in the AHRR and F2RL3 genes are causally involved in lung cancer aetiology by mediating the risk induced by tobacco smoking. Although it is not possible to fully answer this question based on our data, our results are consistent with the notion of a mediating role. We have observed (i) that data from multiple independent study populations have conclusively established an association between tobacco smoking and AHRR and F2RL3 methylation, and (ii) that these methylation sites are also associated with lung cancer risk after adjustment for smoking habits and with careful mediation analysis. Although it is possible that residual confounding from tobacco smoking might still explain the association with risk, we note that the attenuation in OR estimates when adjusting for smoking is negligible in all four studies. Should residual confounding from tobacco smoking explain our observed associations, we would expect a notable attenuation of OR estimates in adjusted risk models. In addition, the observation that smoking-associated hypomethylation in these specific CpG sites is reversible following smoking cessation is compatible with the gradual decrease in lung cancer risk that former smokers experience. A full evaluation of the causal relevance of AHRR and F2RL3 methylation in lung cancer aetiology requires additional investigations, such as a Mendelian randomization analysis of a sufficiently powered study.

Hypomethylation of certain CpG sites/genes, which extends beyond smoking cessation for several years, as observed for the two probes identified in this study, might be more closely associated with lung cancer risk than transient hypomethylation. In previous analyses of healthy subjects, we generally observed a relatively rapid reversal of smoking-related methylation changes, but for a group of probes including cg05575921 and cg03636183 reversal is slower or not apparent even after decades. A larger study is required to evaluate whether reversal of methylation alterations in cg05575921 and cg03636183 occurs at the same rate as the decrease in the risk of lung cancer in former smokers. Also, future prediction models will be built based on a larger number of cohorts not matched by smoking habits (work in preparation). In the present study, we were able to build such a model only for the NOWAC cohort, and there was a modest increase in prediction (AUC changing from 0.71 to 0.78 when methylation information was added).
participants were recruited to the NSHDS in the context of the Västerbotten Intervention Project, which was initiated in 1985 to advocate a healthy diet and lifestyle. All residents in the Västerbottens County were invited to participate in the project by attending a health check-up at 40, 50 and 60 years of age. At the health check-up, which was held at the local health-care centre, participants were asked to complete a self-administered questionnaire including various demographic factors such as education, smoking habits, physical activity and diet. Height and weight were measured and participants were asked to donate a blood sample of 20 ml for future research. Incident lung cancer cases were identified through linkage to the regional cancer registry. Lung cancer cases were defined on the basis of the International Classification of Diseases for Oncology, Second Edition (ICD-O-2), and in most cases primary malignancy was confirmed by physical examination, X-ray and/or histology. As controls, all subsequent steps were performed as described above for NOWAC.

NOWAC laboratory procedures were carried out using in-house software written for the R statistical computing environment. For each sample and each probe, measurements were set to missing if obtained by averaging intensities over less than three beads, or if averaged intensities were below detection thresholds emanating from negative control probes. Background subtraction (to remove hybridization noise and dye bias correction (for probes using the Infinium II design) were also performed. The resulting subset of 473,929 probes targeting autosomal CpG loci was selected for further analyses, and among these, probes with missing values in more than 20% of the samples were excluded, leaving 450,890 probes. Samples with more than 5% of non-detected probes were also excluded from the analysis (14 samples excluded).

DNA methylation data were normalized using a functional normalization procedure that uses the built-in control probes to remove unwanted technical variation. CpG sites that mapped to multiple genomic regions were excluded and CpG sites which had log2-intensity > 0.5 were set to missing. CpG sites were excluded if they were missing in more than 20% of samples. Samples were excluded if more than 5% of their CpG sites were missing or if their average detection P-value was > 0.01. Samples were also dropped if their case-control pair was missing. Of 490 samples initially available, 22 were excluded on the basis of the aforementioned procedures, leaving a total of 234 matched case-control pairs for analysis. Methylation levels at each locus were quantified using the beta-values. In EPIC HD, the quality control measurements included removal of SNP-containing probes, removal of CpGs not analysed in all samples or those in non-CpG context, correction for batch effects and normalization with beta quantile dilution method. DNA methylation profiling with the Illumina Infinium HumanMethylation450 platform. DNA methylation measurement and data pre-processing. Genome-wide DNA methylation analyses were performed on pre-diagnostic blood samples using the Illumina Infinium HumanMethylation450 platform.

NOWAC laboratory procedures were carried out at the University of Bristol, according to the protocol described above for NOWAC. EPIC HD laboratory procedures were carried out at the German Cancer Research Center (DKFZ) and at LGC Bioscience. Buffy coat DNA was isolated at LGC Bioscience by the company’s standardized protocols and returned to DKFZ. DNA methylation profiling with the Illumina Infinium HumanMethylation450 platform was performed at the University of Bristol. DNA methylation measurement and data pre-processing. DNA extraction from lymphocytes and buffy coats was performed using Qiagen mini spin columns, whereas dried blood spot DNA was extracted using a method developed in-house and the quality and quantity of DNA was assessed using the Quant-iT PicoGreen dsDNA assay measured on the Qubit Fluorometer (Life Technologies). Samples were distributed into 96-well plates and processed in chips of 12 arrays (8 chips per plate) with case-control pairs arranged randomly on the same chip. All subsequent steps were performed as described above for NOWAC. NSHDS laboratory procedures were carried out on two sites. DNA extraction from blood samples was performed at the NSHDS laboratory using the FlexiGene DNA Kit (Qiagen GmbH). Illumina Infinium HumanMethylation450 BeadChip analysis was conducted at the NATURE COMMUNICATIONS | DOI: 10.1038/ncomms10192 | www.nature.com/naturecommunications
Mediation analysis. We performed mediation analysis to assess whether methylation of cg05579521 (AHRR) and cg03636183 (F2R3L) probes mediated the effect of smoking (ever smoking versus never smoking) on lung cancer risk using parametric G-computation36 achieved by Monte Carlo simulations39 and adapted to deal with the case–control design following VanderWeele and Vansteelandt40. This requires the specification of a model for the mediator and one for the outcome. Linear regression was used to model methylation levels as a function of smoking status, age and their interaction, and logistic regression to model lung cancer status as a function of age, smoking status, methylation and their interactions. The linear regressions for methylation were weighted to account for the study design; cases were weighted by the prevalence of lung cancer and controls were weighted by 1 minus the prevalence.

We quantified the amount by which either or both of the two methylation probes mediated the effect of smoking on lung cancer incidence by partitioning the TCE of smoking into a NIE and a NDE41,42. We expressed these quantities on the log OR scale because of the case–control design, although they can be interpreted as log rate ratios (because cases are incident lung cancers). The NDE is the effect of smoking on lung cancer (on the log OR scale) when methylation takes the natural value it would have taken in the absence of smoking; whereas the NIE quantifies the change that would be found in log odds of lung cancer for smokers if we could change their methylation level to be that of never smokers. The TCE is the sum of these effects. The proportion of the total effect explained by the hypothesized mechanism (proportion mediated) is given by the ratio between NIE and TCE (on the log scale). Identification of the mediated proportion required structural and parametric assumptions, namely: no unmeasured exposure-mediator, mediator-outcome and exposure-outcome confounding; correct model specification for each of the outcome and the unmeasured exposure-mediator, mediator-outcome and exposure-outcome ratio between NIE and TCE (on the log scale). Identification of the mediated cancer for smokers if we could change their methylation level to be that of never methylation takes the natural value it would have taken in the absence of smoking; log OR scale because of the case–control design, although they can be interpreted in Norway.

In our analysis, it is possible that unmeasured confounders could lead to inaccurate estimates of the effects: in particular, regarding exposure-mediator confounders, information such as smoking intensity, duration of smoking and passive smoking would probably affect the final estimates. The ideal situation would be to create an exposure variable that summarizes all this information and to perform mediation analysis using the new variable as the exposure variable. In our interactions. Air pollution might be a confounder of the repeat mediation analysis using the new variable as the exposure variable. In our smoking status, models were run separately for never, former and current smokers, with adjustment for time since quit smoking (in former smokers) and ORs and 95% CIs were computed.

References
1. Monick, M. M. et al. Coordinated changes in AHRR methylation in lymphoblasts and pulmonary macrophages from smokers. Am. J. Med. Genet. B Neuropsychiatr. Genet. 159B, 141–151 (2012).
2. Elliott, H. R. et al. Differences in smoking associated DNA methylation patterns in South Asians and Europeans. Clin. Epigenetics 6, 4 (2014).
3. Dogan, M. V. et al. The effect of smoking on DNA methylation of peripheral blood mononuclear cells from African American women. BMC Genomics 15, 151 (2014).
4. Zeilinger, S. et al. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. PloS ONE 8, e63812 (2013).
5. Philibert, R. A., Beach, S. R., Lei, M. K. & Brody, G. H. Changes in DNA methylation at the aryl hydrocarbon receptor repressor may be a new biomarker for smoking. Clin. Epigenetics 5, 19 (2013).
6. Besingi, W. & Johansson, A. Smoke-related DNA methylation changes in the etiology of human disease. Hum. Mol. Genet. 23, 2290–2297 (2014).
7. Shenker, N. S. et al. Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking. Hum. Mol. Genet. 22, 843–851 (2013).
8. Philibert, R. A., Beach, S. R. & Brody, G. H. Demethylation of the aryl hydrocarbon receptor repressor as a biomarker for nascent smokers. Epigenetics 7, 1331–1338 (2012).
9. Guida, F. et al. Dynamics of smoking-induced genome-wide methylation changes with time since smoking cessation. Hum. Mol. Genet. 24, 2349–2359 (2015).
10. Ezzati, M. & Lopez, A. D. Estimates of global mortality attributable to smoking in 2000. Lancet 362, 847–852 (2003).
11. Nieminen, C. D. & Loncar, D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med. 3, e42 (2015).
12. Newcomb, P. A. & Carbone, P. P. The health consequences of smoking. Cancer. Med. Clin. North Am. 76, 305 (1992).
13. Vineis, P. et al. Tobacco and cancer: recent epidemiological evidence. J. Natl Cancer Inst. 96, 99–106 (2004).
14. Conen, D. et al. Smoking, smoking cessation, [corrected] and risk for symptomatic peripheral artery disease in women: a cohort study. Ann. Intern. Med. 154, 719–726 (2011).
15. Kawachi, I. et al. Smoking cessation and decreased risk of stroke in women. JAMA 269, 232–236 (1993).
16. Vermeulen, R. et al. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptor II increases are increased in sputum. Am. J. Respir. Crit. Care Med. 166, 1218–1224 (2002).
17. Willems, B. W. et al. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. Eur. Respir. J. 26, 835–845 (2005).
18. Ebbert, J. O. et al. Lung cancer risk reduction after smoking cessation: observations from a prospective cohort of women. J. Clin. Oncol. 21, 921–926 (2003).
19. Vermeulen, R. & Chadeau-Hyam, M. Dynamic aspects of exposure history—do they matter? Epidemiology 23, 900–906 (2012).
20. Vlaanderen, J. et al. Effect modification of the association of cumulative exposure and cancer risk by intensity of exposure and time since exposure cessation: a flexible method applied to cigarette smoking and lung cancer in the SYNERGY Study. Am. J. Epidemiol. 179, 290–297 (2014).
21. Zhang, Y., Yang, R., Burwinkel, B., Breitling, L. P. & Brenner, H. F2R3L methylation as a biomarker of current and lifetime smoking exposures. Environ. Health Perspect. 122, 131–137 (2014).
22. Wan, E. S. et al. Cigarette smoking behaviors and time since quitting are associated with differential DNA methylation across the human genome. Hum. Mol. Genet. 21, 3073–3082 (2012).
23. Talikka, M. et al. Genomic impact of cigarette smoke, with application to three smoking-related diseases. Crit. Rev. Toxicol. 42, 877–889 (2012).
24. Vineis, P., Schatzkin, A. & Potter, J. D. Models of carcinogenesis: an overview. Carcinogenesis 31, 1703 (2010).
25. Hankinson, O. The aryl hydrocarbon receptor complex. Annu. Rev. Pharmacol. Toxicol. 35, 307 (1995).
26. Zhang, Y. et al. F2R3L methylation in blood DNA is a strong predictor of mortality. Int. J. Epidemiol. 43, 1215–1225 (2014).
27. Relton, C. L. & Davey Smith, G. Two-step epigenetic Mendelian randomization: a strategy for establishing the causal role of epigenetic processes in pathways to disease. Int. J. Epidemiol. 41, 161–176 (2012).
28. Giles, G. & Enghlish, D. The Melbourne Collaborative Cohort Study. IARC Sci. Publ. 156, 69 (2002).
29. Hallmans, G. et al. Cardiovascular disease and diabetes in the Northern Sweden Health and Disease Study Cohort—evaluation of risk factors and their interactions. Scand. J. Public Health Suppl. 61, 18–24 (2003).
30. Riboli, E. & Kaaks, R. The EPIC Project: Rationale and Study Design. Int. J. Epidemiol. 26(Suppl 1): S6–S14 (1997).
31. Boeing, H., Wahrenberg, J. & Becker, N. EPIC-Germany—A source for studies into diet and risk of chronic diseases. European Investigation into Cancer and Nutrition. Ann. Nutr. Metab. 43, 195–204 (1999).
32. Joo, J. E. et al. The use of DNA from archival dried blood spots with the InfinumHumanMethylation450 array. BMC Biotechnol. 13, 23 (2013).
33. Maksimovic, J., Gordon, L. & Oshlack, A. SWAN: Subset-quantile within array normalization for illumina Infinium HumanMethylation450 BeadChips. Genome Biol. 13, R44 (2012).
34. Fortin, J. P. et al. Functional normalization of 45K methylation array data improves replication in large cancer studies. Genome Biol. 15, 503 (2014).
35. Naeem, H. et al. Reducing the risk of false discovery enabling identification of biologically significant genome-wide methylation status using the HumanMethylation450 array. BMC Genomics 15, 51 (2014).
36. Du, P. et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics 11, 587 (2010).
37. Housman, E. A. et al. DNA methylation arrays as surrogate measures of cell population distribution. BMC Bioinformatics 13, 86 (2012).
38. Robins, J. A new approach to causal inference in mortality studies with a sustained exposure period—application to control of the healthy worker survivor effect. Math. Modelling 7, 1393 (1986).
39. Daniel, R., De Stavola, B. & Cousens, S. gformula: Estimating causal effects in a dichotomous outcome. Ann. Epidemiol. 172, 1339–1348 (2010).
Acknowledgements
The NOWAC postgenome cohort study has been funded by the ERC advanced grant ‘Transcriptomics in Cancer Epidemiology—ERC-2008-AdG-232997’. The methylation analyses for the NOWAC cohort were performed at the Human Genetics Foundation in Torino, Italy with funds from Compagnia di San Paol. The MCCS nested case–control study of methylation and lung cancer risk was funded by a grant from the Australian National Health and Medical Research Council (Project Grant #1050198). For the MCCS, we acknowledge the contribution of Dr Chol-hee Jung and Dr Jessica Chung from the Life Sciences Computation Centre and the Victorian Life Sciences Computation Initiative in Melbourne for the preparation of the bioinformatics pipeline and Drs Ming Wong and Jihoon Joo for their contribution to the methylation analyses conducted in the Genetic Epidemiology Laboratory, Department of Pathology, the University of Melbourne. The NSHDS nested case–control study of methylation and lung cancer risk was funded by a grant from the Roy Castle Lung Cancer Foundation and supported by the MRC Integrative Epidemiology Unit at the University of Bristol (MC_UU_12013_2). We are indebted to the laboratory and bioinformatics team (Dr Wendy McArdle, Dr Nabila Kazmi, Dr Hashem Shihab and Dr Tom Gaunt) for their contribution to the generation of the Illumina Infinium HumanMethylation450 data for this analysis. The EPIC-Heidelberg cohort study was conducted with the financial support of the ‘Europe Against Cancer’ program of the European Commission (SANCO), the German Cancer Aid (Deutsche Krebshilfe) and the German Federal Ministry of Education and Research (BMBF). The present nested case–control study of methylation and lung cancer risk was supported by the German Center for Lung Research (DZL), grant PB13394. L.B. is supported by a Marie Curie International Incoming Fellowship within the 7th European Community Framework Programme.

Authors contributions
All authors participated in the conception and writing of manuscript

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Fasanelli, F. et al. Hypomethylation of smoking–related genes is associated with future lung cancer in four prospective cohorts. Nat. Commun. 6:10192 doi: 10.1038/ncomms10192 (2015).

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