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# DNA methylation changes associated with cancer risk factors and blood levels of vitamin metabolites in a prospective study

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Aberrant DNA methylation is a major epigenetic mechanism of gene silencing in a wide range of human cancers. Previous studies on DNA methylation typically used paired tumor and normal-appearing surrounding tissues from cancer-bearing individuals. However, genomic DNA isolated from surrogate tissues such as blood cells represents an attractive material that can be exploited in the discovery of biomarkers of exposure and tumorigenesis. Here we examined the association between lung cancer and DNA methylation patterns in a panel of candidate genes. We also investigated whether blood levels of vitamin metabolites modify DNA methylation levels in blood cells. To this end, we quantitatively determined DNA methylation levels in blood cells of nested cases and controls from a prospective study with well defined dietary habits and lifestyles. Multiple CpG sites in five genes (CDKN2A/p16, RASSF1A, GSTP1, MTHFR and MGMT) that are frequent targets of hypermethylation in a variety of human malignancies were included in the analysis. While no clear association between DNA methylation patterns and the case/control status was found, with the exception of RASSF1A hypermethylation, methylation level changed according to serum levels of 1-carbon metabolites and vitamins B. Overall, folate was associated with increased methylation levels of RASSF1A and MTHFR and methionine was associated with decreased methylation levels of RASSF1A. The associations with folate were more pronounced among never smokers while the associations with methionine were more evident among ever-smokers. These results are consistent with the notion that blood levels of 1-carbon metabolism markers and dietary/lifestyle factors may modify DNA methylation levels in blood cells and that blood cells can be exploited for the discovery of epigenetic biomarkers of exposures, providing proof-of-principle on the use of blood samples in the context of prospective studies.



## Introduction

Lung cancer continues to be the most common cancer and leading cause of cancer death worldwide. The vast majority of lung cancer cases are due to tobacco smoking, with a striking dose-response relationship.[1] The disease has proven difficult to diagnose early and treat successfully, reflecting limited advances in the identification of cancer biomarkers. This is largely due to our limited understanding of the molecular mechanisms underlying lung carcinogenesis and individual susceptibility to lung cancer.

In addition to genetic changes, including mutations and genetic polymorphisms, epigenetic alterations have been strongly implicated in lung cancer pathogenesis. Aberrant DNA methylation is a major epigenetic mechanism of gene silencing in a wide range of human cancers, including lung cancer.[2,3] DNA methylation-mediated silencing, either alone or in combination with genetic alterations, may lead to the inactivation of tumor suppressor genes and other cancer-associated genes promoting lung cancer. A number of recent studies have identified aberrant CpG methylation of several genes in lung cancer,[4-6] either in single cancer-associated genes, or in multiple gene promoters.[7] These studies provide a strong support for a critical role of DNA methylation patterns and epigenetic gene silencing in the development and progression of lung cancer.

Previous studies on DNA methylation in lung cancer typically used paired tumor and normal-appearing surrounding tissues from cancer-bearing individuals. The use of surrogate tissues including DNA from blood cells has been relatively little explored. Blood DNA methylation levels in specific genomic regions (including LINE-1 and Alu repetitive elements) were shown to undergo rapid changes in response to

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**Table 1.** Characteristics of cases and controls

| | Controls (N) | Cases (N) |
|---|---|---|
| **Total** | 99 | 93 |
| Men | 49 | 46 |
| Women | 50 | 47 |
| **Education** | | |
| None | 6 | 5 |
| Primary School Completed | 30 | 37 |
| Technical/Professional school | 22 | 18 |
| Secondary school | 20 | 19 |
| University | 17 | 8 |
| Not specified/missing | 4 | 6 |
| **Country** | | |
| France | 4 | 1 |
| Italy | 24 | 25 |
| Spain | 15 | 15 |
| UK | 19 | 19 |
| The Netherlands | 15 | 12 |
| Germany | 22 | 21 |
| **Smoking Status** | | |
| Never | 31 | 30 |
| Former | 30 | 31 |
| Current | 38 | 32 |
| **Drinking Status** | | |
| None drinker | 4 | 6 |
| Former Drinker | 5 | 6 |
| Current Drinker | 85 | 75 |
| unknown | 5 | 6 |
| **BMI** | | |
| <18.5 | 3 | 0 |
| 18.5–25 | 43 | 36 |
| 25–30 | 40 | 46 |
| ≥30 | 13 | 11 |
| **Age at blood draw** | 59 | 59 |
| **Histology** | | |
| Squamous Cell Carcinoma | | 17 |
| Adenocarcinoma | | 36 |
| Small Cell Carcinoma | | 12 |
| Large Cell Carcinoma | | 3 |
| Other/unspecified | | 25 |

environmental factors,[8] suggesting that DNA methylation levels and patterns in blood DNA may serve as biomarkers of exposure. Also, as one-carbon metabolism provides methyl groups for methylation, relevant metabolites in body fluids may be associated with methylation levels in target cells. Whether surrogate tissues harbour changes in DNA methylation associated with lung cancer, and whether such changes are associated with one-carbon metabolites has not yet been fully investigated. We selected the genes that may have an association with lung cancer based on their supposed biological function and the genes that were proposed to be frequent targets of hypermethylation in cancer or that are involved in the DNA methylation process itself.

In the present paper we describe a prospective study in which methylation patterns in candidate genes and one-carbon metabolites have been investigated in lung cancer patients and a set of controls, using pre-diagnostic blood samples (with a lag time between blood drawing and cancer diagnosis).

## Results

**Analysis of DNA methylation patterns of CDKN2A/p16, RASSF1A, GSTP1, MTHFR and MGMT genes in blood samples from lung cancer cases and controls.** Multiple CpG sites in the promoter region of five genes (CDKN2A/p16, RASSF1A, GSTP1, MTHFR and MGMT) that are frequent targets of aberrant hypermethylation in a variety of human malignancies were selected for the analysis. All the regions included in the analysis are in bona fide CpG islands and pyrosequencing assays have already been reported.[5] General characteristics of cancer cases and controls from the EPIC cohort included in this study are shown in **Table 1**. The results of the pyrosequencing analysis are shown in **Table 2**. We assessed possible associations between DNA methylation levels and lung cancer risk by performing an unconditional logistic regression analysis. No clear association was found between methylation patterns in peripheral blood cells and the case/control status, but statistical power was limited. Only RASSF1A was associated with lung cancer in a borderline statistical fashion (**Table 2**). We also conducted stratified analysis by time since blood drawing. As shown in **Table 3**, lung cancer was associated with hypermethylated RASSF1A among subjects with >8 years of follow-up. A weaker association was found with CDKN2/p16 hypermethylation, whereas DNA methylation levels in MGMT, GSTP1 and MTHFR were not associated with lung cancer risk by time since blood drawing.

**Association between DNA methylation levels and 1-carbon metabolism.** The process of DNA methylation requires dietary methyl donors and can be influenced by environmental and dietary/lifestyle factors.[2,3,19-21] Therefore, we next investigated whether DNA methylation levels changed according to serum levels of 1-carbon metabolites and B vitamins. **Table 4** shows the changes in DNA methylation levels for increasing vitamin plasma levels (with 95% confidence intervals). We found that folate levels were associated with increasing methylation levels of both RASSF1A and MTHFR in never smokers. Methionine was associated exclusively with decreasing methylation levels, in CDKN2A/p16, RASSF1A and MTHFR in former smokers and RASSF1A and GSTP1 in current smokers. Homocysteine concentration was associated with decreased methylation of MTHFR in current smokers, while vitamin $B_{12}$ showed opposite associations with RASSF1A (decreased methylation in former smokers) and MTHFR (increased methylation in current smokers). These results suggest that blood levels of 1-carbon metabolism markers and dietary/lifestyle factors may modify

**Table 2.** Odds ratios (OR) and 95% confidence interval (CI) for methylation levels (below/above median in controls) and lung cancer risks

| | Controls | Cases | Crude OR | 95% CI | Adjusted OR[1] | 95% CI |
|---|---|---|---|---|---|---|
| **CDKN2A/P16 (Tumor Suppressor)** | | | | | | |
| 0 | 54 | 49 | 1.00 | | 1.00 | |
| >0 | 44 | 43 | 1.08 | (0.61–1.91) | 0.99 | (0.54–1.81) |
| **RASSF1A (Tumor Suppressor)** | | | | | | |
| <1.82 | 53 | 38 | 1.00 | | 1.00 | |
| ≥1.82 | 45 | 54 | 1.67 | (0.94–2.97) | 1.87 | (1.00–3.49) |
| **Tertiles** | | | | | | |
| ≤1.77 | 34 | 26 | 1.00 | | 1.00 | |
| 1.77–1.87 | 30 | 28 | 1.22 | (0.59–2.52) | 1.30 | (0.59–2.86) |
| >1.87 | 34 | 38 | 1.46 | (0.73–2.91) | 1.57 | (0.75–3.30) |
| P for trend | | | 0.28 | | 0.23 | |
| **MGMT (DNA Repair)** | | | | | | |
| <8.55 | 49 | 52 | 1.00 | | 1.00 | |
| ≥8.55 | 49 | 41 | 0.79 | (0.45–1.39) | 0.77 | (0.42–1.41) |
| **GSTP1 (Detoxification)** | | | | | | |
| <0.97 | 49 | 44 | 1.00 | | 1.00 | |
| ≥0.97 | 47 | 49 | 1.16 | (0.66–2.06) | 1.24 | (0.66–2.34) |
| **MTHFR (Folate Metabolism)** | | | | | | |
| <1.89 | 48 | 50 | 1.00 | | 1.00 | |
| ≥1.89 | 49 | 43 | 0.84 | (0.48–1.49) | 0.91 | (0.49–1.67) |
| **Combined 5 markers** | | | | | | |
| 0–2 | 46 | 48 | 1.00 | | 1.00 | |
| 3–4 | 47 | 39 | 0.80 | (0.44–1.43) | 0.81 | (0.44–1.50) |
| 5 | 5 | 6 | 1.15 | (0.33–4.03) | 1.17 | (0.28–4.81) |
| ever 1 marker | | | 1.04 | (0.86–1.27) | 1.04 | (0.84–1.05) |

[1]Models were adjusted for age at blood draw, sex, country, education (no degree/primary school, technical or professional school, secondary school, university degree, and not specified/missing), cotinine concentration in serum (as a surrogate for smoking status), baseline alcohol drinking (continuous), and body mass index (BMI, continuous).

DNA methylation levels in gene-specific manner in peripheral blood cells.

## Discussion

In the present study, we observed that higher levels of methylation in RASSF1A and CDKN2/p16 were associated with lung cancer risk by time since blood drawing, whereas DNA methylation levels in MGMT, GSTP1 and MTHFR were not associated with cancer risk. There is growing evidence suggesting that changes in target tissues during cancer development and progression and in tumor cells may also be reflected in the epigenotype of peripheral blood cells.[22] An analogous phenomenon has been reported for peripheral-blood transcriptome as surrogate for cancer prediction.[23,24] While a larger study in genome-wide settings may be needed to identify a DNA methylation signature in peripheral blood cells that could reliably predict the disease and classify cancer patients and healthy individuals, our study serves as proof of principle that the epigenotype of peripheral blood cells may be associated with cancer risk.

The precise mechanism that underlies targeting of specific genes (such as RASSF1A) for hypermethylation in peripheral blood cells of individuals susceptible to lung cancer remains unclear. Generally, it has been proposed that different factors involved in the process of DNA methylation, such as DNMT activity and proximity to a methylation center, as well as locus-specific factors (such as transcription factor motifs) and local histone marks, could be involved in the differential susceptibility to DNA methylation among the genes.[25] In this respect, it is noteworthy that among the genes analyzed in our study, RASSF1A and CDKN2A/p16 have been frequently found hypermethylated in lung cancer. Our observations may also reflect a higher susceptibility of different tissues (both target and surrogate) to hypermethylation in individuals at high risk of cancer. It is thus tempting to speculate that the mechanism underlying a common deregulation of DNA methylation machinery may occur across different cell types and tissues in susceptible individuals. Alternatively, a susceptibility to the deregulation of the factors involved in protecting against unscheduled DNA methylation may occur in multiple tissues and tissue-specific factors may further modulate the magnitude of DNA methylation. Further

**Table 3.** Odds ratios (OR) and 95% confidence intervals (CI) for methylation levels (below/above median in controls) and lung cancer risk by time since blood drawing

| | ≤8 years | | | | >8 years | | | |
|---|---|---|---|---|---|---|---|---|
| | Controls | Cases | OR | 95% CI | Controls | Cases | OR | 95% CI |
| **CDKN2A/p16 (Tumor Suppressor)** | | | | | | | | |
| 0 | 6 | 41 | 1.00 | | 48 | 8 | 1.00 | |
| >0 | 9 | 28 | 0.42 | (0.08–2.19) | 35 | 15 | 2.02 | (0.71–5.77) |
| **RASSF1A (Tumor Suppressor)** | | | | | | | | |
| <1.82 | 5 | 31 | 1.00 | | 48 | 7 | 1.00 | |
| ≥1.82 | 10 | 38 | 0.51 | (0.11–2.39) | 35 | 16 | 2.91 | (0.98–8.61) |
| **MGMT (DNA Repair)** | | | | | | | | |
| <8.55 | 6 | 38 | 1.00 | | 43 | 14 | 1.00 | |
| ≥8.55 | 9 | 31 | 0.27 | (0.05–1.44) | 40 | 10 | 0.75 | (0.28–2.03) |
| **GSTP1 (Detoxification)** | | | | | | | | |
| <0.97 | 7 | 35 | 1.00 | | 42 | 9 | 1.00 | |
| ≥0.97 | 8 | 34 | 0.99 | (0.25–3.98) | 39 | 15 | 1.65 | (0.53–5.13) |
| **MTHFR (Folate Metabolism)** | | | | | | | | |
| <1.89 | 5 | 41 | 1.00 | | 43 | 9 | 1.00 | |
| ≥1.89 | 10 | 28 | 0.13 | (0.02–0.78) | 39 | 15 | 1.88 | (0.67–5.29) |
| **Combined 5 markers** | | | | | | | | |
| 0–2 | 4 | 40 | 1.00 | | 42 | 8 | 1.00 | |
| 3–4 | 10 | 27 | 0.18 | (0.04–0.91) | 37 | 12 | 1.72 | (0.59–5.02) |
| 5 | 1 | 2 | 0.16 | (0.01–5.24) | 4 | 4 | 3.84 | (0.62–23.9) |
| ever 1 marker | | | 0.55 | (0.30–1.00) | | | 1.31 | (0.92–1.88) |

studies are required to elucidate a possible common mechanism underlying preferential targeting of specific genes in target and surrogate tissues.

Unscheduled DNA hypermethylation of promoter regions acts to silence genes and affects key cellular processes, including cell growth and proliferation, DNA repair and cell death. Most of the gene targets of aberrant hypermethylation were identified based on the candidate gene approach. A well-studied example in lung cancer is the hypermethylation of the tumor suppressor gene CDKN2A/p16, which correlates with gene silencing and is an early event in tumorigenesis,[5,26-28] supporting our observation that the association is stronger in the strata with longer follow-up. CDKN2A/p16 hypermethylation might be less important after a tumor is initiated. In addition, the candidate tumor suppressor gene RASSF1A was frequently found hypermethylated in lung cancer in previous studies.[29,30] Also for RASSF1A the association in our study was more pronounced among subjects followed-up for more than 8 years. Combining the observation on the number of hypermethylated genes and follow-up years might suggest that methylation is a cumulative event and might be involved in different stages of carcinogenesis. Aberrant DNA methylation has also been found in genes that cannot be considered classical tumor suppressor genes or cancer associated genes. For example, DNA methylation changes of MTHFR gene have been found in lung cancer.[5] It is therefore likely that DNA methylation changes may alter expression of genes with weak or no tumor suppressing activity, including genes with cellular functions such as DNA repair.

Our findings that DNA methylation changes at the genes analyzed in blood lymphocytes can predict lung cancer risk is intriguing. Notably, it is unclear how epigenetic changes in the genes that are not directly related to lung cancer may contribute to and/or predict the development of lung tumors. It is possible that epigenetic deregulation of these genes and their corresponding pathways in cells with hematopoietic and immune system functions may elicit common changes indirectly in other cell types such as in the lung epithelium. For example, altered expression of specific genes in lymphocytes may alter their immune response that, in combination with other risk factor exposures, may result in changes in lung tissues and ultimately contribute to cancer susceptibility. Alternatively DNA methylation alterations in these genes may simply reflect changes in blood lymphocyte composition in individuals at high risk. However, whether changes in DNA methylation in these genes may be causally involved in susceptibility to lung cancer must await further investigations. Nevertheless, DNA methylation changes in blood lymphocytes may prove to be bona fide lung cancer-predisposition markers.

Another interesting observation is that DNA methylation levels in specific genes might be associated with blood levels of 1-carbon metabolites and B vitamins. For example, folate levels were associated with increased methylation in RASSF1A and MTHFR, whereas methionine levels were associated with decreased methylation in RASSF1A. Several associations were tested using this set of about 180 cases and controls and some of

**Table 4.** Association[1] between vitamin B/1-carbon metabolites and methylation level by smoking status

| | | CDKN2A/p16 | | | RASSF1A | | | MGMT | | | GSTP1 | | | MTHFR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | unit | β | lcl | ucl | β | lcl | ucl | β | lcl | ucl | β | lcl | ucl | β | lcl | ucl |
| Folate | 10 | 0.04 | -0.05 | 0.14 | **0.05** | **0.01** | **0.10** | 0.09 | -0.39 | 0.61 | 0.05 | -0.04 | 0.15 | -0.06 | -0.18 | 0.06 |
| Vitamin $B_{12}$ | 100 | -0.01 | -0.03 | 0.01 | -0.01 | -0.02 | 0.01 | 0.01 | -0.12 | 0.22 | -0.01 | -0.03 | 0.02 | 0.00 | -0.03 | 0.05 |
| Homocysteine | 10 | 0.02 | -0.17 | 0.22 | 0.01 | -0.11 | 0.12 | -0.11 | -1.27 | 1.16 | 0.00 | -0.21 | 0.23 | 0.18 | -0.11 | 0.49 |
| Methionine | 10 | -0.06 | -0.15 | 0.05 | **-0.06** | **-0.11** | **0.00** | -0.22 | -0.82 | 0.45 | -0.02 | -0.12 | 0.10 | -0.08 | -0.20 | 0.06 |
| Vitamin $B_2$ | 10 | 0.00 | -0.01 | 0.00 | 0.00 | 0.00 | 0.01 | -0.01 | -0.04 | 0.05 | 0.00 | 0.00 | 0.02 | 0.00 | -0.01 | 0.01 |
| Vitamin $B_6$ | 10 | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 | 0.01 | -0.01 | -0.07 | 0.07 | 0.00 | -0.01 | 0.01 | 0.00 | -0.01 | 0.01 |
| **Never Smokers** | | | | | | | | | | | | | | | | |
| Folate | 10 | 0.06 | -0.12 | 0.25 | **0.15** | **0.02** | **0.29** | 0.21 | -0.81 | 1.28 | 0.08 | -0.11 | 0.29 | 0.13 | -0.07 | 0.34 |
| Vitamin $B_{12}$ | 100 | -0.01 | -0.03 | 0.04 | -0.01 | -0.04 | 0.02 | 0.04 | -0.11 | 0.29 | 0.00 | -0.03 | 0.04 | 0.02 | -0.02 | 0.07 |
| Homocysteine | 10 | 0.30 | -0.32 | 0.97 | 0.31 | -0.19 | 0.81 | 0.24 | -4.02 | 4.53 | **0.87** | **0.19** | **1.59** | -0.01 | -0.75 | 0.74 |
| Methionine | 10 | -0.04 | -0.21 | 0.18 | -0.09 | -0.23 | 0.06 | -0.73 | -1.66 | 0.36 | 0.28 | 0.01 | 0.60 | 0.11 | -0.12 | 0.39 |
| Vitamin $B_2$ | 10 | 0.00 | -0.10 | 0.01 | 0.00 | 0.00 | 0.01 | -0.01 | -0.05 | 0.05 | 0.00 | -0.01 | 0.01 | 0.00 | -0.01 | 0.02 |
| Vitamin $B_6$ | 10 | 0.02 | -0.02 | 0.07 | -0.01 | -0.04 | 0.03 | 0.15 | -0.10 | 0.42 | 0.01 | -0.04 | 0.06 | 0.02 | -0.03 | 0.08 |
| **Former Smokers** | | | | | | | | | | | | | | | | |
| Folate | 10 | -0.03 | -0.14 | 0.10 | 0.01 | -0.02 | 0.04 | 0.27 | -0.44 | 1.06 | 0.08 | -0.05 | 0.23 | -0.11 | -0.26 | 0.05 |
| Vitamin $B_{12}$ | 100 | **-0.11** | **-0.21** | **-0.02** | **-0.03** | **-0.06** | **0.00** | 0.09 | -0.53 | 0.79 | -0.10 | -0.22 | 0.03 | -0.04 | -0.17 | 0.11 |
| Homocysteine | 10 | -0.11 | -0.34 | 0.15 | -0.01 | -0.08 | 0.06 | -0.08 | -1.54 | 1.56 | 0.02 | -0.26 | 0.32 | 0.12 | -0.23 | 0.50 |
| Methionine | 10 | **-0.20** | **-0.37** | **-0.02** | **-0.06** | **-0.11** | **0.00** | -0.71 | -1.97 | 0.63 | -0.02 | -0.21 | 0.20 | **-0.43** | **-0.64** | **-0.22** |
| Vitamin $B_2$ | 10 | -0.01 | -0.03 | 0.01 | 0.00 | -0.01 | 0.01 | 0.00 | -0.14 | 0.18 | 0.01 | -0.02 | 0.05 | 0.00 | -0.03 | 0.04 |
| Vitamin $B_6$ | 10 | **-0.03** | **-0.05** | **-0.01** | 0.00 | -0.01 | 0.01 | -0.07 | -0.22 | 0.10 | -0.01 | -0.04 | 0.03 | -0.03 | -0.06 | 0.00 |
| **Current Smokers** | | | | | | | | | | | | | | | | |
| Folate | 10 | 0.03 | -0.14 | 0.20 | -0.03 | -0.08 | 0.03 | -0.54 | -1.61 | 0.60 | -0.01 | -0.17 | 0.15 | -0.19 | -0.39 | 0.02 |
| Vitamin $B_{12}$ | 100 | 0.03 | -0.09 | 0.15 | 0.02 | -0.02 | 0.06 | 0.54 | -0.34 | 1.42 | -0.03 | -0.16 | 0.11 | 0.04 | -0.13 | 0.21 |
| Homocysteine | 10 | 0.10 | -0.21 | 0.43 | -0.03 | -0.14 | 0.08 | -1.02 | -3.38 | 1.65 | **-0.35** | **-0.68** | **0.00** | 0.28 | -0.16 | 0.75 |
| Methionine | 10 | -0.01 | -0.17 | 0.16 | **-0.07** | **-0.12** | **-0.02** | 0.16 | -1.05 | 1.50 | **-0.21** | **-0.36** | **-0.05** | 0.14 | -0.06 | 0.35 |
| Vitamin $B_2$ | 10 | -0.01 | -0.05 | 0.04 | 0.00 | -0.02 | 0.01 | -0.14 | -0.45 | 0.28 | -0.02 | -0.06 | 0.03 | -0.04 | -0.10 | 0.04 |
| Vitamin $B_6$ | 10 | 0.01 | 0.00 | 0.03 | 0.00 | 0.00 | 0.01 | -0.04 | -0.09 | 0.04 | 0.00 | -0.01 | 0.01 | 0.00 | -0.01 | 0.02 |

[1]The models were adjusted for age at blood drawing, sex, country, education (no degree/primary school, technical or professional school, secondary school, university degree, and not specified/missing), cotinine concentration in serum (continuous), baseline alcohol drinking (continuous), BMI (continuous) and case-control status. Abbreviations: β, regression coefficients; lcl, lower confidence interval; ucl, upper confidence interval; Units for metabolites: folate (nmol/L), vitamin B12 (pmol/L), homocysteine (μmol/L), methionine (μmol/L), vitamin B2 (nmol/L) and vitamin B6 (nmol/L).

the observed associations could be chance findings. On the other hand, the 1-carbon metabolism pathway is the main conduit for methyl group donation at a cellular level; therefore, our study might provide support to the notion that serum levels of 1-carbon metabolites have an impact on DNA methylation of different genes in peripheral blood cells. Our results are also consistent with the idea that DNA methylation levels are associated with dietary and lifestyle factors.

Diet influences DNA methylation levels in cells in several ways, but mainly via the 1-carbon metabolism pathway. Folate, methionine and vitamin B6 deficiency have been associated with an increased risk of cancer at different sites, including lung.[31] Our results further support the concept that plasma levels of 1-carbon metabolites and B vitamins could influence the methylation level of several genes. However, the precise mechanism that underlies preferential modulation of DNA methylation levels of a specific set of genes by 1-carbon metabolites and B vitamins remains enigmatic. Recent studies suggested that DNA methylation is a much more dynamic process than previously assumed. Studies on the activation of the Estrogen Receptor (ER) pathway found that specific genes undergo activation cycles resulting from rapid cycles of DNA methylation/demethylation.[32,33] DNA methyltransferases appear to exhibit dual actions during these cycles and periodic, strand-specific methylation/demethylation may occur during transcriptional activation of specific genes. It is thus possible that specific genes may be more vulnerable to deficiency in dietary folate and other vitamins and consequent changes in plasma levels of 1-carbon metabolites.

In summary, our study nested in a longitudinal cohort provides proof-of-principle for the use of blood samples to investigate DNA methylation patterns related to lung cancer risk and dietary factors. We have also provided evidence of a modulatory effect of plasma concentrations of 1-carbon metabolites/B vitamins on methylation patterns. Together our results support the notion that DNA methylation in blood cells may act as a surrogate for cancer risk and environmental exposure. Further studies in high-throughput and genome-wide settings may potentially provide key information for epigenetic tests with the sensitivity and specificity required for lung cancer risk assessment and the development of preventive strategies.

## Methods

**The EPIC study.** The European Prospective Investigation into Cancer and Nutrition (EPIC) is an ongoing study aiming to investigate the relationship between diet, lifestyle and environmental factors and the incidence of cancer at various sites.[9,10] The EPIC cohort was initiated in 1992 and has grown into a multicentre study on a large number of participants aged 35–70 years, among 23 centres in 10 European countries: Denmark, France, Germany, Greece, Italy, the Netherlands, Spain, Norway, Sweden and the United Kingdom. The study centres and target populations were selected from the general population of specific geographic areas, towns or provinces in order to have diversity in the levels of exposure and cancer frequency. Therefore, comparisons can be made between populations with heterogeneous dietary habits and lifestyles and with different rates of cancer occurrence. After obtaining ethical approval from the IARC IRB and from the local Ethical Committees at the participating centres, participants were invited to take part in the study. The questionnaires aimed at gathering lifestyle and personal history data and included questions on habitual diet, education, employment, current and past occupation that might have led to carcinogen exposure, active and environmental tobacco smoke, contraceptive and reproductive history, use of hormone replacement therapy, physical activity, history of previous and/or current illnesses, any medical and surgical treatment and hospitalization.[9,10]

**Follow up and case ascertainment.** New cancer cases (including lung cancer) among the EPIC participants are identified through the cancer registries in seven of the participating countries (Denmark, Italy, The Netherlands, Norway, Spain, Sweden and the United Kingdom). In France, Germany and Greece, a combination of methods is used including cancer and pathology registries, health insurance records[9,10] and active follow up of study participants and their next-of-kin. Data on total and cause-specific mortality are obtained either through cancer mortality registries or via active follow up. Incident cancer cases are coded according to the International Classification of Diseases-Oncology 2nd Edition (ICD-O-2) (www. dimdi.de/static/en/klassi/diagnosen/icdo3/index.htm). At the latest follow-up more than 2,200 incident cases of lung cancer accrued in EPIC, including 1,800 with blood samples.

**Nested case-control study.** Lymphocyte DNA samples were obtained from a previous study on genetic susceptibility and environmental factors in non-smokers (GenAIR),[11] a case-control study nested within the EPIC cohort,[9,10] to which a group of current smokers has been added. Overall, we have used samples from 93 subjects with lung cancer diagnosed after recruitment in EPIC, with an average follow-up of 6.0 years. Cases were randomly selected, being approximately one third never smokers, one third former smokers and one third current smokers. Controls have been matched for age, sex, year of recruitment, smoking (never, ex, current) and country using incidence density sampling (i.e., selected from available potential controls at the time of lung cancer diagnosis in the case). Originally 100 cases had been selected, but for 7 cases and 1 control DNA was not available. Buffy coat samples were retrieved from liquid nitrogen at IARC. DNA was extracted for all subjects. Data on plasma folate and B-vitamins were already available from a previous study in the same population,[12] and were linked to the methylation results and the epidemiological dataset.

**Blood lymphocyte samples and DNA extraction.** Time elapsed between blood collection and straw preparation was within 24 h (except for one centre), generally 2–5 h. Straws were kept at -80°C for 24–72 h and then stored in liquid nitrogen. Genomic DNA from lymphocytes was extracted using buffy coat separated from blood samples by automated equipment (Autopure LS by Gentra Systems) as described previously,[5] and DNA concentrations were quantified with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and with Quant'iT PicoGreen dsDNA reagent (Molecular Probes) as described previously.[5,13]

**Bisulfite conversion and DNA methylation analysis by pyrosequencing.** The methylation level in the selected panel of genes[5] was examined by pyrosequencing, a highly reliable and quantitative method for the analysis of DNA methylation at multiple CpG sites with built-in internal controls for completeness of bisulfite treatment.[14-16] Bisulfite treatment was carried out as described previously.[5] Genomic DNAs (0.5–1 μg) from blood lymphocytes were treated with EZ DNA methylation-Gold kit (Zymo Research, USA), according to the manufacturer's protocol. The modified DNA samples (20–25 ng/μL) were stored at -20°C until use. In order to investigate the methylation level of several genes, targeted sequences were localized in bona fide CpG islands.[17,18] For each gene, sets of primers were designed on an in silico modified DNA sequence (**Suppl. Table 1**). DNA amplifications were carried out on bisulfite-treated DNA using specific primers and PCR conditions.[5] Modified DNAs (20–25 ng) were amplified in a total volume of 50 μL. 10 μL of PCR reaction were analyzed on agarose gel, and the remaining 40 μL were used in pyrosequencing assay using sequencing primers. Pyrosequencing reactions were set up using PyroGold Reagent kit (Biotage, Sweden) according to the manufacturer's instructions. The methylation levels at the target CpGs were evaluated by converting the resulting pyrograms to numerical values for peak heights and expressed either as percentage of methylation of individual CpG sites or as the mean of all CpG analyzed at a given gene promoter.[5]

**Exposure variables.** Detailed information on lifetime history of consumption of tobacco products was assessed by means of questions on smoking status (current, past or never smoker), type of tobacco used (cigarettes, cigars or pipe), number of cigarettes currently smoked, age when participants started and, if applicable, quit smoking. Dietary intake assessment was carried out by extensive country-specific dietary questionnaires, aiming to provide high compliance rates and to detect between and within country variations in dietary habits.[9,10]

**Biochemical analysis.** Measurements of plasma concentrations of vitamin $B_2$, vitamin $B_6$, folate, vitamin $B_{12}$, cotinine, total homocysteine and methionine were performed at Bevital A/S (www.bevital.no) in a blinded way as to case-control status.[12]

**Statistical analyses.** The associations between DNA methylation levels and lung cancer risk were modelled by an unconditional logistic regression model. Hypermethylation is defined by the median of the methylation level among the controls. All models included the following variables: age at blood drawing, sex, country (categorical), education (no degree/primary school, technical or professional school, secondary school, university degree and not specified/missing), cotinine concentration in serum, baseline alcohol drinking (continuous) and body mass index (BMI, continuous). Because epigenetic changes could be an early event of carcinogenesis, we stratified the analysis at 8 years of follow-up, based on a reasonably balanced distribution of the cases and controls. The associations between vitamin B metabolites and DNA methylation levels were modeled by generalized linear regression models and assumed that the dependent variables (DNA methylation levels, theoretical range: 0–100) were following a γ-distribution with an identity link function. To fit the γ-distribution assumption, DNA methylation levels were inflated by adding 1 to all values. All analyses were performed using SAS 9.1. All tests were two sided and statistical significance was assessed at the level of 0.05.

## Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/Vineis-EPI6-2-Sup.pdf

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