

## ARTICLE

# Modulation of Age- and Cancer-Associated DNA Methylation Change in the Healthy Colon by Aspirin and Lifestyle

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Manuscript received September 2, 2013; revised April 23, 2014; accepted May 12, 2014.

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**Background** Aberrant DNA methylation in gene promoters is associated with aging and cancer, but the circumstances determining methylation change are unknown. We investigated the impact of lifestyle modulators of colorectal cancer (CRC) risk on the stability of gene promoter methylation in the colonic mucosa.

**Methods** We measured genome-wide promoter CpG methylation in normal colon biopsies ( $n = 1092$ ) from a female screening cohort, investigated the interaction of lifestyle factors with age-dependent increase in methylation with log-linear multivariable regression, and related their modifying effect to hypermethylation in CRC. All statistical tests were two-sided.

**Results** Of 20025 promoter-associated CpGs analyzed, 1713 showed statistically significant age-dependent methylation gains. Fewer CpGs acquired methylation in users of aspirin ( $\geq 2$  years) and hormonal replacement therapy (HRT age  $\geq 50$  years) compared with nonusers (43 vs 1355; 1 vs 1377, respectively), whereas more CpGs were affected in smokers ( $\geq 20$  years) and individuals with a body mass index (BMI) of  $25 \text{ kg/m}^2$  and greater compared with control groups (180 vs 39; 554 vs 144, respectively). Fifty percent of the CpGs showing age-dependent methylation were found hypermethylated in CRC (odds ratio [OR] = 20; 95% confidence interval [CI] = 18 to 23;  $P < 2 \times 10^{-16}$ ). These loci gained methylation with a higher median rate compared with age-only methylated sites ( $P = 2 \times 10^{-76}$ ) and were enriched for polycomb regions (OR = 3.67). Importantly, aspirin ( $P < .001$ ) and HRT use ( $P < .001$ ) reduced the methylation rate at these cancer-related genes, whereas smoking ( $P < .001$ ) and high BMI ( $P = .004$ ) increased it.

**Conclusions** Lifestyle, including aspirin use, modulates age-associated DNA methylation change in the colonic epithelium and thereby impacts the evolution of cancer methylomes.

JNCI J Natl Cancer Inst (2014) 106(7): dju161 doi:10.1093/jnci/dju161

Aberrations in DNA methylation deregulate the genome and contribute to the loss of tissue homeostasis observed in aging and diseases such as cancer (1,2). They can constitute both driver and passenger events of tumorigenesis, as evident from widespread changes in CpG methylation observed in cancers. Among those, the hypermethylation of promoter-associated CpG islands is a well-documented phenomenon (3), often associated with the loss of expression of the respective gene (4). Exactly why and how such epigenetic change arises is currently unknown.

Hypermethylation at promoters of tumor suppressor genes is a hallmark of colorectal cancer (CRC), for which age is a major risk factor (5,6). An increasing body of evidence indicates that aging is, at the same time, associated with an accumulation of aberrations in DNA methylation in human tissues, including colon (7–10). We and others have shown that de novo methylation at CpG island promoters of cancer-relevant genes arise in the aging mucosa of healthy individuals with no evidence of intestinal disease (11,12). Notably, these methylation changes appeared in patterns implicating a contribution of

physiological and environmental factors (11,13,14). Understanding what controls the stability of DNA methylation in aging tissue is of both biological and clinical importance. Herein we address to what extent age-associated methylation change in the human colon is influenced by lifestyle and whether the ensuing epigenetic aberrations have relevance for neoplastic transformation. For this purpose, we determined CpG methylation levels in normal colon biopsies from a large female screening cohort and related these to the exposure of well-accepted CRC risk modulators (15–19): aspirin use, hormone replacement therapy (HRT), BMI, and smoking habits.

## Methods

### Study Participants and Data Collection

The study included 546 healthy women randomly recruited from a national colonoscopy screening for CRC in Poland (20). Ethical approval was obtained for all samples. From each participant, biopsies of normal mucosa from the cecum and sigmoid colon were

collected in RNA<sup>Later</sup> (Applied Biosystems, Lucerne, Switzerland) and stored at  $-80^{\circ}\text{C}$ . Information on the use of aspirin, HRT, and cigarette smoking was collected using a self-administered questionnaire under the assistance of a study nurse (Table 1; Supplementary Materials, available online).

### Quantification of DNA Methylation

**Locus-Specific DNA Methylation.** Bisulfite-converted genomic DNA of 1092 samples was used to measure human MutL homolog 1 (*bMLH1*) and O<sup>6</sup>-methylguanine DNA methyltransferase (*MGMT*) promoter methylation levels (percentage of methylated alleles) by locus normalized quantitative methylation specific polymerase chain reaction (ln-qMSP). Full details are available in the Supplementary Materials (available online). Primer sequences are provided in the Supplementary Table 1.

**Genome-Wide DNA Methylation.** Genome-wide assessment of DNA methylation was done on Infinium HumanMethylation27 Beadchip arrays (Illumina, San Diego, CA), interrogating methylation at 27 578 CpGs distributed in the promoters of 14 475 coding genes. Details on quantitation of methylation levels are available in the Supplementary Materials (available online). Microarray data were validated by bisulfite pyrosequencing (Supplementary Materials, available online).

All primary data generated in the study were deposited in the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>; accession No. GSE48988. Methylation data of 59 female CRC samples were obtained from GEO (accession No. GSE25062) (21).

### Gene Expression Analysis

Expression data for 32 colon adenomas/normal tissues were obtained from GEO (accession No. GSE8671) (22). The data were median normalized, log<sub>2</sub> transformed, and analyzed using the R/Bioconductor limma package.

### H3K27me3 Status in Human Embryonic Stem Cells

H3K27me3 is a polycomb repressive mark of interest because its presence in stem cells has been shown to predispose genomic sequences to aberrant DNA methylation during aging and carcinogenesis (8,23,24). An H3K27me3 profile in human embryonic stem cells was obtained from GSE13084 (25). Five hundred base-pair windows symmetrically positioned around the Illumina Infinium HumanMethylation27 CpGs were analyzed for enrichment of H3K27me3.

### Statistical Analyses

**Locus-Specific DNA Methylation Analysis.** Log-linear multivariable regression models were applied to investigate the interaction of age-related DNA methylation changes with lifestyle factors by considering age as a linear predictor and the four lifestyle factors of aspirin use, HRT use, BMI, and smoking as categorical predictors. To analyze methylation interaction with age, corresponding interaction terms were introduced in the model. To investigate the interaction of age with polyps, separate multivariable regression models (adjusted for the interaction of the four lifestyle factors with age) were done by considering age as a linear predictor and polyps as categorical predictors (either as one group [any polyp] or classified as tubular adenomas

**Table 1.** Clinical characteristics of the study population

Characteristic	No. of subjects (%)
Age, y	
<60	275 (50)
≥ 60	271 (50)
Aspirin regular use*	
Nonuser†	391 (71)
Short-term user (< 2y)‡	86 (16)
Long-term user (≥ 2y)§	69 (19)
Hormone replacement therapy	
Nonuser¶	245 (45)
Aged <50 y#	175 (32)
Aged ≥ 50 y**	126 (23)
Body mass index, kg/m <sup>2</sup> ††	
Normal, 18.5–25	227 (42)
Overweight, 26–30	224 (41)
Obese, >30	95 (17)
Cigarette smoking‡‡	
Nonsmoker§§	272 (50)
Short-term smoker, <20 y	122 (22)
Long-term smoker, ≥20 y¶¶	152 (28)
Polyps	
No polyps	350 (64)
Yes	196 (36)
Proximal colon###	
Tubular adenoma	49 (25)
Serrated lesion***	25 (13)
Hyperplastic polyps	22 (11)
Sessile serrated adenoma	3 (2)
Distal colon†††	
Tubular adenoma	41 (21)
Serrated lesion	81 (41)
Hyperplastic polyps	69 (35)
Sessile serrated adenoma	12 (6)

\* Regular use defined as two or more tablets per week for 1 or more months.

† Non-user: women who indicated that they did not use two or more aspirin tablets per week for 1 or more months (minimum level).

‡ Short-term user: women who indicated that they used two or more aspirin tablets per week for less than 2 years.

§ Long-term user: women who indicated that they used two or more aspirin tablets per week for 2 or more years.

|| Hormone replacement therapy defined as estrogen therapy and/or oral contraceptive for 1 or more years.

¶ Non-user: women who indicated that they did not have hormone replacement therapy for 1 or more years (minimum level).

# Aged <50: women who indicated that they did had hormone replacement therapy for 1 or more years before age 50 years.

\*\* Aged ≥50 years: women who indicated that they did have hormone replacement therapy for 1 or more years after the age of 50 years.

†† Body mass index: height (cm) and weight (kg) were self-reported, and body mass index was calculated (kg/m<sup>2</sup>) from these variables.

‡‡ Cigarette smoking was defined as one or more cigarettes per day for one or more years.

§§ Nonsmoker: women who indicated that they did not smoke one or more cigarettes per day for 1 or more years (minimum level).

||| Short-term smoker: women who indicated that they smoked one or more cigarettes per day for less than 20 years.

¶¶ Long-term smoker: women who indicated that they smoked one or more cigarettes per day for 20 or more years.

### Proximal colon: cecum.

\*\*\* Serrated lesion: any serrated polyp including hyperplastic and sessile serrated adenoma.

††† Distal colon: sigmoid.

and serrated lesions, including both hyperplastic polyps and sessile serrated adenomas). Separate calculations were done for each colonic location (proximal and distal colon) and gene. For data presentation, log-linear regression coefficients were back transformed and used to calculate rates of change in methylation per 10-year increase in age in respective lifestyle strata. The fold difference in the rate of change in methylation between two categories was defined as the methylation rate ratio (MRR). Full details and analyses are given in the [Supplementary Materials](#) (available online) and [Supplementary Tables 2–4](#) (available online). The association between lifestyle factors and occurrence of polyps was examined by means of logistic multivariable regression models adjusted for age, aspirin use, HRT use, BMI, and smoking but without age interaction ([Supplementary Table 5](#), available online). Regression coefficients are expressed as odds ratios (ORs). Median regression analysis was conducted as supplementary analysis to evaluate the impact of extreme values on the results. The impact of methylation values below the detection limit was tested with tobit regression. Neither supplementary analysis changed the results, and thus they are not reported.

**Genome-Wide DNA Methylation Analysis.** The differential analysis of methylation data was performed on the *M* value (log<sub>2</sub> ratios of methylated and unmethylated probes) using routines implemented in the R/Bioconductor *limma* package (26). Full details are given in the [Supplementary Materials](#) (available online). False-discovery rate (FDR)-adjusted *P* values for multiple comparison were calculated using the Benjamini and Hochberg approach. CpGs were considered statistically significantly differentially methylated with age when displaying an FDR-adjusted *P* value less than .05. CpGs were considered statistically significantly hypermethylated in colorectal cancer vs normal when displaying FDR-adjusted *P* value less than .0001 and fold change difference in *M* values greater than 2. *P* values for the difference in median rate of DNA methylation change were calculated by Wilcoxon rank sum test. *P* values and ORs for overlap were calculated using Fisher exact test. Cancer relevance of identified age-related hypermethylation targets was addressed using the tumor-associated genes (TAG) database (27). Pathway analyses were performed with KEGGprofile in R/Bioconductor. All statistical tests were two-sided.

## Results

We investigated a cohort of 546 healthy women aged 50 to 80 years for relationships between gene promoter methylation in the colonic mucosa and the reported use of aspirin, reported HRT, BMI, and smoking ([Table 1](#)).

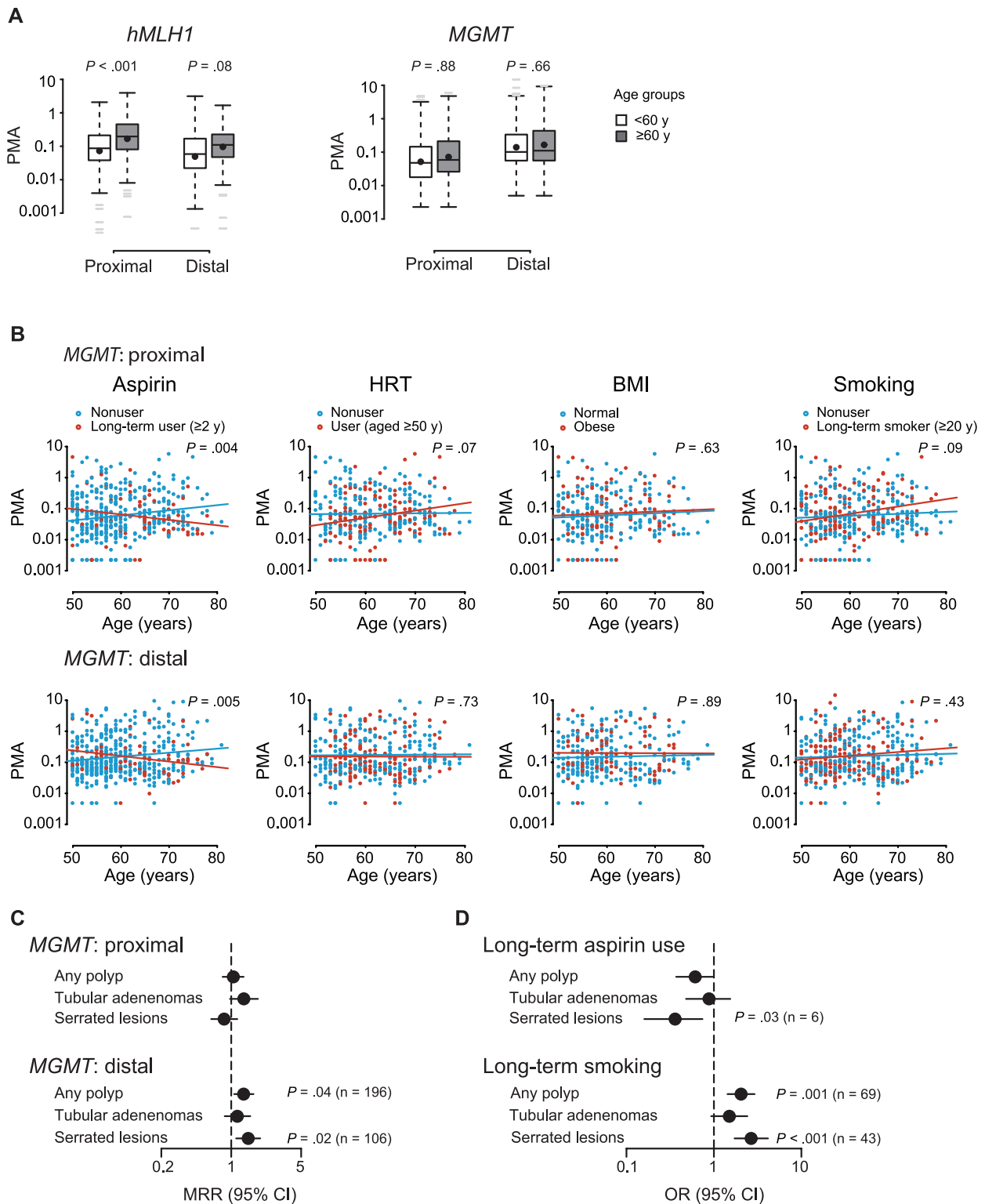
### Age-Related DNA Methylation and Association With Lifestyle and Polyp Occurrence

We first measured *bMLH1* and *MGMT* promoter methylation as percentage of methylated alleles by ln-qMSP in a total of 1092 normal biopsies obtained from cecum (proximal) and sigmoid (distal) colon. Log-linear multivariable regression analysis showed that methylation at the *bMLH1* promoter statistically increased with age in the proximal colon (rate per 10 years of age = 2.1%; *P* < .001) ([Figure 1A](#); [Supplementary Table 2](#), available online). By contrast, the *MGMT* promoter did not show an overall age-dependent methylation change ([Figure 1A](#); [Supplementary Table 3](#), available online) unless lifestyle

factors were taken into account ([Figure 1B](#); [Supplementary Table 3](#), available online); long-term aspirin use was associated with a more than 50% suppressed rate of methylation when compared with non-use (proximal colon: MRR = 0.44, 95% CI = 0.27 to 0.70, *P* = .004; distal colon: MRR = 0.47, 95% CI = 0.31 to 0.73, *P* = .005), whereas HRT after the age of 50 years and long-term smoking slightly stimulated *MGMT* methylation in the proximal colon (HRT: MRR = 1.63, 95% CI = 1.05 to 2.53, *P* = .07; smoking: MRR = 1.51, 95% CI = 1.01 to 2.26, *P* = .09). Notably, women with serrated lesions had a 48% higher rate of *MGMT* promoter methylation in the distal colon compared with women with no polyps (MRR = 1.48; 95% CI = 1.12 to 1.95; *P* = .02) ([Figure 1C](#); [Supplementary Table 4](#), available online), and the risk of serrated lesions was reduced in long-term aspirin users (OR = 0.36; 95% CI = 0.16 to 0.74; *P* = .03) but increased in long-term smokers (OR = 2.67; 95% CI = 1.72 to 4.15; *P* < .001) ([Figure 1D](#); [Supplementary Table 5](#), available online). No association with lifestyle and/or the occurrence of polyps was found for *bMLH1* promoter methylation ([Supplementary Table 2](#), available online).

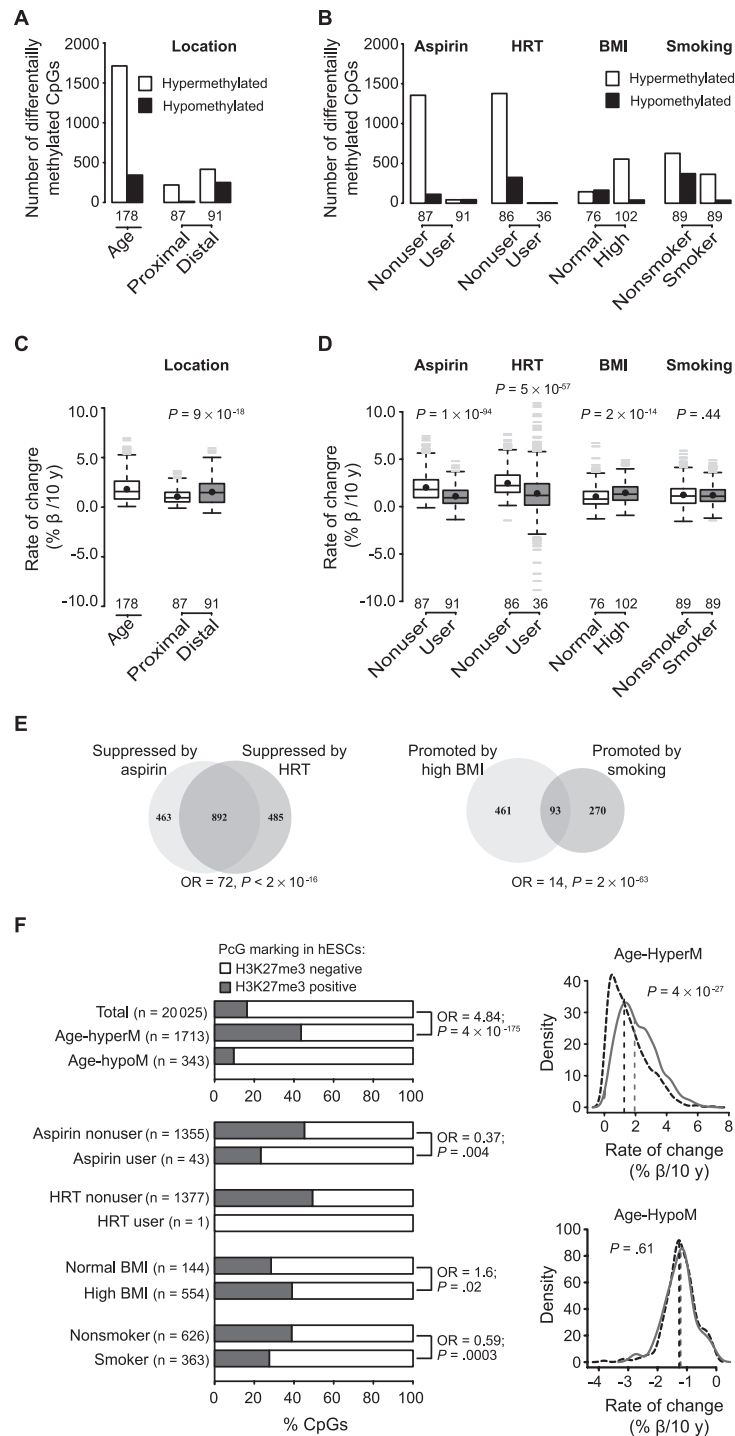
Next, we analyzed genome-wide DNA methylation in 178 normal colon samples from our cohort (Illumina Infinium HumanMethylation27 BeadChip). We analyzed 20025 promoter-associated CpGs with multivariable regression adjusted for colonic location, aspirin use, HRT, BMI, and smoking and identified 1713 CpGs gaining and 343 losing methylation with age; 219 CpGs acquired methylation exclusively in the proximal, and 416 acquired methylation exclusively in the distal colon ([Figure 2A](#)). When stratified for lifestyle factors, age-dependent hypermethylation was suppressed in aspirin users (43 CpGs in users vs 1355 in nonusers) and/or in women reporting HRT (1 CpG in users vs 1377 in nonusers) but promoted in individuals with a high BMI (554 CpGs in high BMI vs 144 in normal BMI) ([Figure 2B](#)). Whereas aspirin use, HRT, and BMI modulated age-dependent DNA methylation changes along the entire colon, the effect of smoking was confined to the proximal colon (180 CpGs in smokers vs 39 in nonsmokers) ([Supplementary Figure 2](#), A and B, available online). The median rate of age-dependent methylation change at hypermethylated CpGs (median rate = 1.6%; range = 0.07%–6.97%) was 56% higher in the distal colon than in the proximal colon (MRR = 1.56; *P* =  $9 \times 10^{-18}$ ) ([Figure 2C](#)). The rate was 48% lower in aspirin users and 47% lower in women reporting HRT compared with non-users (aspirin: MRR = 0.52, *P* =  $1 \times 10^{-94}$ ; HRT: MRR = 0.53; *P* =  $5 \times 10^{-37}$ ) ([Figure 2D](#)), whereas a high BMI was associated with a 27% increased methylation rate in the distal colon (MRR = 1.27; *P* =  $5 \times 10^{-9}$ ), and smoking was associated with a 400% increased rate in the proximal colon (MRR = 4.67; *P* =  $1 \times 10^{-28}$ ) but a 33% decreased rate in the distal colon (MRR = 0.66; *P* =  $8 \times 10^{-46}$ ) ([Supplementary Figure 2](#), A and B, available online).

Examining potential interactions between these lifestyle effects, we found statistically significant overlaps between CpGs showing suppression of age-related methylation by aspirin use or HRT (OR = 72; 95% CI = 62 to 83; *P* <  $2 \times 10^{-16}$ ) ([Figure 2E](#)) and, less pronounced, between CpGs showing BMI- or smoking-stimulated hypermethylation (OR = 14; 95% CI = 11 to 19; *P* =  $2 \times 10^{-63}$ ). Although the methylation-retarding effect of aspirin use was apparent in the entire study population, it appeared more effective in individuals with a high BMI (52%; MRR = 0.48; *P* =  $2 \times 10^{-31}$ ) compared with individuals with a normal BMI (22%; MRR = 0.78; *P* = .0001)



**Figure 1.** Association of human MutL homolog 1 (*hMLH1*) and O<sup>6</sup>-methylguanine DNA methyltransferase (*MGMT*) promoter methylation with lifestyle factors and polyps. **A** *hMLH1* and *MGMT* promoter methylation in proximal (cecum) and distal (sigmoid) colon as percentages of methylated alleles (PMAs) determined by locus normalized quantitative methylation specific polymerase chain reaction (ln-qMSP). For presentation only, age is shown in two groups as indicated. Shown are median (lines) and mean (black circles) PMAs with interquartile ranges (boxes), 1.5 times the interquartile ranges (whiskers), and extreme values (gray lines). **B** Association between age-dependent *MGMT* promoter methylation and lifestyle factors. Each point represents one

biopsy. *P* values are derived from log-linear multivariable regression analysis (Supplementary Table 3, available online) representing significance of the difference in two regression lines. **C** Association of *MGMT* promoter methylation with the occurrence of polyps. Methylation rate ratios (MRRs) and *P* values are derived from log-linear multivariable regression (Supplementary Table 4, available online). **D** Association of lifestyle parameters with the occurrence of polyps. Odds ratios (ORs) and *P* values are derived from logistic multivariable regression analysis (Supplementary Table 5, available online). BMI = body mass index; CI = confidence interval; HRT = hormone replacement therapy. All statistical tests were two-sided.



**Figure 2.** Genome-wide DNA methylation and its association with lifestyle factors. **A** and **B**) Numbers of age-associated differentially methylated CpGs in all samples or when stratified by colon location (proximal [cecum] vs distal [sigmoid]) and lifestyle factors (aspirin: nonuser vs user [long-term]; hormone replacement therapy [HRT]: nonuser vs user [aged  $\geq 50$  years]; body mass index [BMI]: normal vs high [ $>25 \text{ kg/m}^2$ ]; smoking: nonsmoker vs smoker [long-term]). Numbers of samples tested in each category are indicated at the bottom of each bar. **C** and **D**) Ten-year rates of DNA methylation change for CpGs showing age-associated hypermethylation in all samples or when stratified by colon location and lifestyle factors. Shown are median (lines) and mean (black circles) rates with interquartile ranges (boxes), 1.5 times the interquartile ranges (whiskers), and extreme values (gray lines).  $P$  values according to the Wilcoxon rank sum test. **E**) Concordance of probes showing suppression of age-associated

methylation by aspirin-use and HRT or promotion of age-associated methylation by a high BMI and long-term smoking.  $P$  values and odds ratios (ORs) according to the Fisher exact test. **F**) Enrichment of age-associated differentially methylated sites marked by histone 3 lysine 27 tri-methylation (H3K27me3) in human embryonic stem cells (hESCs). Barplots indicate percentages of age-associated hypermethylated (Age-hyperM) and hypomethylated (Age-hypoM) CpGs either marked by H3K27me3 (positive) or not (negative), or the enrichment of CpGs marked by H3K27me3 in lifestyle modulated age-related hypermethylation (bottom). Odds ratios and  $P$  values according to Fisher exact test. Density plots on the right show the rate of change in Age-hyperM and Age-hypoM probes at H3K27me3 positive (gray line) and negative (black, dashed line) CpGs. Dashed vertical lines indicate median rate of change per 10 years of age. PcG = polycomb group. All statistical tests were two-sided.

or in smokers (79%; MRR = 0.21;  $P = 3 \times 10^{-20}$ ) compared with non-smokers (31%; MRR = 0.69;  $P = 2 \times 10^{-84}$ ) (Supplementary Figure 2C, available online). HRT use retarded methylation in individuals with a high BMI only (70%; MRR = 0.30;  $P = 9 \times 10^{-44}$ ), whereas its effect on smoking remained unclear because of limited statistical power.

### Association of Lifestyle-Modulated DNA Methylation With Genomic Regions Disposed to Hypermethylation in Cancer

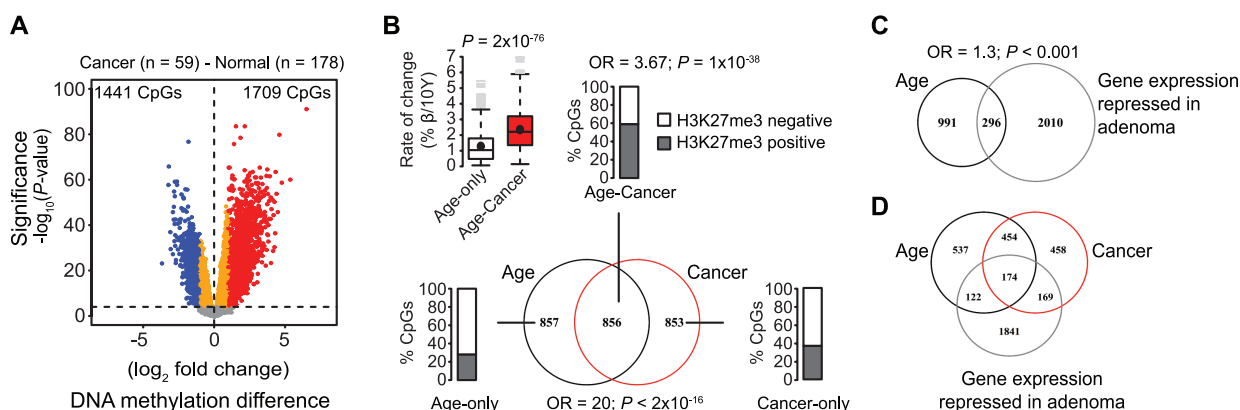
To investigate whether CpGs prone to age-dependent methylation are distinguished by specific genomic features, we classified array CpG probes with regard to coincidence of H3K27me3 in human embryonic stem cells. Regions surrounding age-hypermethylated CpGs were statistically significantly enriched for domains marked by H3K27me3 in stem cells compared with total H3K27me3 marked CpGs on the array (OR = 4.84; 95% CI = 4 to 5;  $P = 4 \times 10^{-175}$ ) (Figure 2F), and the median rate of age-dependent methylation was 53% higher at these sites compared with sites depleted for H3K27me3 (MRR = 1.53;  $P = 4 \times 10^{-27}$ ). Aspirin use suppressed hypermethylation selectively at H3K27me3 marked sequences (user vs nonuser: OR = 0.37; 95% CI = 0.2 to 0.8;  $P = .004$ ), whereas a high BMI promoted methylation at these sites (high vs normal BMI: OR = 1.6; 95% CI = 1.1 to 2.5;  $P = .02$ ). The association of hypermethylation with H3K27me3 modification was less in smokers (smoker vs nonsmokers: OR = 0.59; 95% CI = 0.5 to 0.8;  $P = .0003$ ), indicating that smoking promotes methylation in CpG-rich regions other than the typical polycomb targets.

### Association of Lifestyle-Modulated DNA Methylation with CRC

To address the cancer relevance of these age- and lifestyle-modulated DNA methylation changes in the healthy colon,

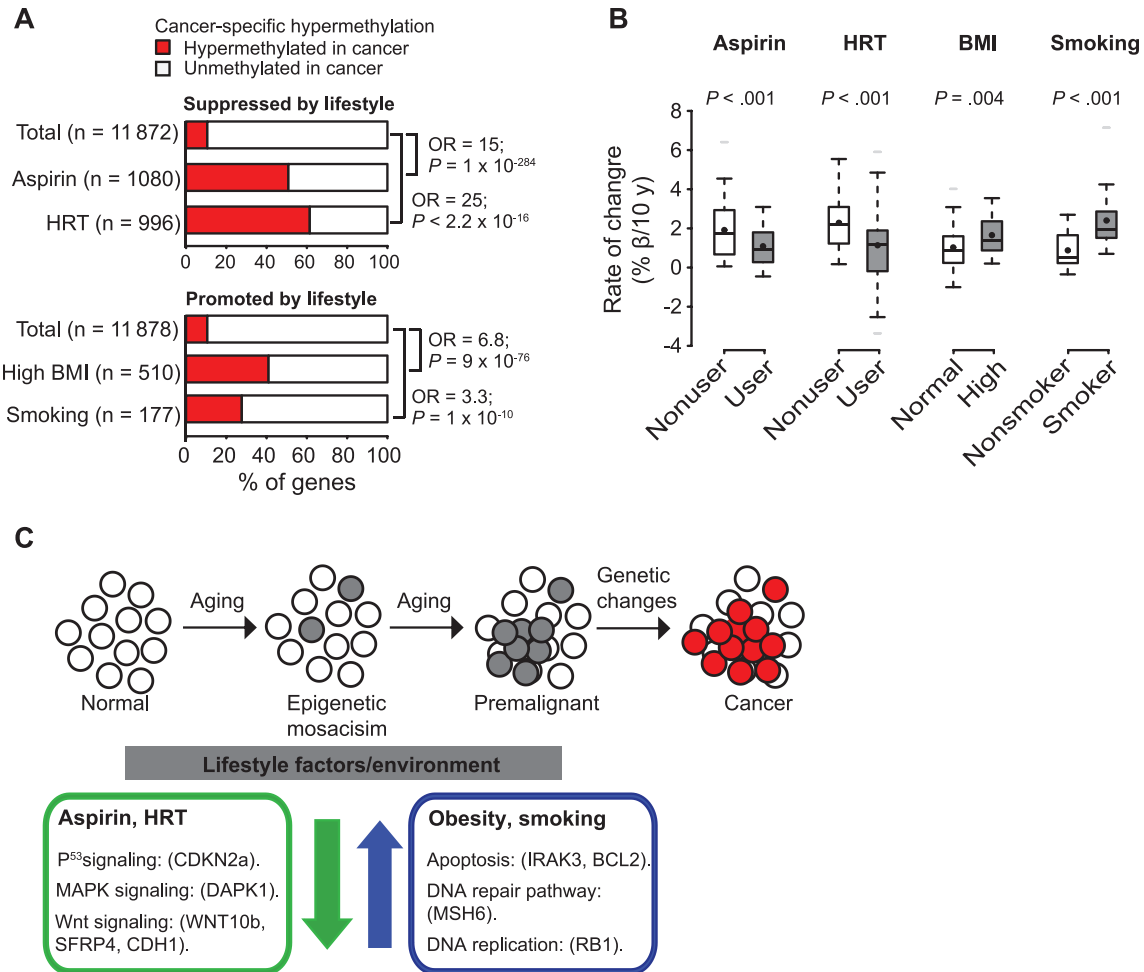
we compared methylation data of 59 CRCs (female patients) included in a genome-wide methylation study (21) with those of our healthy mucosa samples. We identified 1709 CpGs statistically significantly hypermethylated and 1441 CpGs hypomethylated in the CRCs (Figure 3A). Half of the CpGs showing age-dependent hypermethylation in the healthy mucosa ( $n = 856$  of 1713; OR = 20; 95% CI = 18 to 23;  $P < 2 \times 10^{-16}$ ) coincided with sites hypermethylated in CRC (Figure 3B; Age-Cancer). At these loci, the median rate of methylation gain per 10 years was doubled (MRR = 2.21;  $P = 2 \times 10^{-76}$ ) compared with age-methylated sites only (Figure 3B; Age-only), and they were most highly enriched for polycomb regions (OR = 3.67; 95% CI = 3.0 to 4.5;  $P = 1 \times 10^{-38}$ ). Age-hypermethylated CpGs also coincided partially with genes transcriptionally downregulated ( $n = 296$  of 1287) (Figure 3C) in colonic adenomas (22), and 174 genes showed all features (ie, age- and CRC-dependent hypermethylation and transcriptional downregulation in adenomas) (Figure 3D).

Importantly, cancer-relevant methylation change in the normal colonic mucosa was influenced by lifestyle. A statistically significant fraction of promoters showing either aspirin- or HRT-suppressed methylation (aspirin:  $n = 549$  of 1080, OR = 15, 95% CI = 13 to 17,  $P = 1 \times 10^{-284}$ ; HRT:  $n = 612$  of 996, OR = 25, 95% CI = 22 to 30,  $P < 2.2 \times 10^{-16}$ ) or BMI- or smoking-promoted methylation (high BMI:  $n = 209$  of 510, OR = 6.8, 95% CI = 6 to 8,  $P = 9 \times 10^{-76}$ ; smoking in proximal colon:  $n = 49$  of 177, OR = 3.3, 95% CI = 2 to 7,  $P = 1 \times 10^{-10}$ ) in the aging colon coincided with sites hypermethylated in CRC (Figure 4A). These included genes controlling key aspects of carcinogenesis (Supplementary Figures 3–6, available online) such as cell cycle regulation (*CDKN2A*), DNA repair (*MGMT*), apoptosis (*DAPK1*), cell invasion (*CDH1*), and Wnt (*WNT16*) and RAS signaling (*RASSF1*) (2,28–30). Moreover, the rates of methylation change at promoters of established



**Figure 3.** Enrichment of colorectal cancer (CRC)-associated hypermethylation in age-related methylated genes. **A**) Differences in DNA methylation between 59 CRC samples (21) of female patients and 178 normal biopsies. Plotted are difference in log<sub>2</sub>-fold change (FC) in DNA methylation on the x-axis with false discovery rate (FDR)-adjusted  $P$  values (calculated by moderated  $t$  statistics;  $-1 \times \log_{10}$  scale) on the y-axis. CpGs statistically significantly hypermethylated in CRC are highlighted in red ( $n = 1709$ ; FDR-adjusted  $P < .0001$ ; FC > 2). **B**) Intersection between age-related hypermethylated CpGs in healthy mucosa and CpGs hypermethylated in tumor samples. Barplots indicate percentages of CpGs marked by histone 3 lysine 27 tri-methylation (H3K27me3) in each intersection (Age-only, Age-Cancer, Age-only, Cancer-only). The boxplot shows median rates of DNA methylation change per 10 years

for age-tumor vs age-only hypermethylated loci. Shown are median (lines) and mean (black circles) rates with interquartile ranges (boxes), 1.5 times the interquartile ranges (whiskers), and extreme values (gray lines). **C**) Intersection between 1287 age-related hypermethylated genes ( $n = 1713$  CpGs) in the normal colon mucosa and genes downregulated (FDR-adjusted  $P \leq .05$ ) in colon adenomas (22). **D**) Intersection between genes statistically significantly hypermethylated over age in the normal colon mucosa, genes hypermethylated in CRC samples, and genes downregulated in colon adenomas. Odds ratios (ORs) and associated  $P$  values were calculated according to the Fisher exact test.  $P$  values for the difference in median rates of DNA methylation change were calculated according to the Wilcoxon rank sum test. All statistical tests were two-sided.



**Figure 4.** Modulation of cancer-associated hypermethylation by lifestyle factors. **A**) Concordance between genes showing modulation of age-associated methylation by lifestyle parameters and hypermethylation in colorectal cancer (CRC). Enrichment in each category is calculated over the percentage of 11 872 genes ( $n = 20\,025$  CpGs) present on the Illumina array (Total). Odds ratios (ORs) and  $P$  values were calculated according to the Fisher exact test.  $n$  = number of genes. **B**) Median rates of DNA methylation on 664 annotated tumor-associated genes (TAG database) (27).  $n$  = number of tumor-associated genes hypermethylated in each category.  $P$  values are calculated by Wilcoxon rank sum test. Shown are median (lines) and mean (black circles) rates with interquartile ranges

(boxes), 1.5 times the interquartile ranges (whiskers), and extreme values (gray lines). **C**) Mode for the modulation of CRC risk by lifestyle factors (41). Inherent DNA methylation instability generates epigenetic mosaicism (gray cells) in the aging colonic epithelium. Some methylation changes will affect transcription of genes controlling carcinogenesis, eventually contributing to the evolution of premalignant (gray cell cluster) and cancer cells (red cell cluster). Lifestyle factors are capable of negatively (blue arrow) or positively (green arrow) influencing progressive age-related methylation change, thereby connecting lifestyle with cancer risk. BMI = body mass index; HRT = hormone replacement therapy; OR = odds ratio. All statistical tests were two-sided.

tumor-associated genes (27) were statistically significantly reduced in aspirin users or in women reporting HRT compared with nonusers (aspirin:  $MRR = 0.53$ ,  $P < .001$ ; HRT:  $MRR = 0.54$ ,  $P < .001$ ) (Figure 4B) but increased in smokers vs nonsmokers or in individuals with high BMI vs normal BMI (smoking in proximal colon:  $MRR = 4.0$ ,  $P < .001$ ; high BMI:  $MRR = 1.57$ ,  $P = .004$ ).

## Discussion

We report that lifestyle factors that are known modulators of CRC risk have widespread effects on the stability of gene promoter methylation in the aging colonic mucosa. We found age-associated DNA hypermethylation to be suppressed by regular aspirin use and HRT use but accelerated by long-term smoking and a high BMI, which is concordant with the effects of these factors on CRC risk. Statistically significant subsets of affected promoters were

associated with genes controlling key aspects of carcinogenesis and with chromatin features known to predispose to hypermethylation in cancer, accentuating the cancer relevance of the lifestyle-modulated DNA methylation change in the normal colon. These findings provide an important resource for understanding the interplay between lifestyle exposure and aging in the modulation of epigenetic (in)stability in colonic epithelial cells and, thereby, in the evolution of CRC methylomes.

Our data extend the well-established relationships between aging and gene promoter hypermethylation (9,31,32) by providing a functional link to cancer. Comparing genome-wide methylation data of CRC (21) with those of normal aging mucosa allowed us to discriminate between age-only, cancer-only and age-cancer hypermethylation. Importantly, the rate of methylation change at age-cancer hypermethylated sites was statistically significantly higher than at loci showing age-only methylation, and aspirin use and

HRT use suppressed whereas high BMI and smoking promoted methylation in a large fraction of these promoters. The identification of this specific subgroup of gene promoters, displaying age-associated and cancer-associated hypermethylation modulated by lifestyle factors, is important for several reasons. First, the targets provide insight into molecular pathogenesis of CRC and how lifestyle factors may exert their effects on CRC risk. Second, age-associated hypermethylation at such targets in the normal colonic mucosa implies a risk for CRC. Hence, age-cancer hypermethylated CpGs may serve as biomarkers for risk prediction and disease progression because their methylation can be monitored in the healthy mucosa. Third, unlike genetic mutations, epigenetic alterations are reversible and the identification of gene promoters at which hypermethylation can be modulated by lifestyle will help develop tailored methylation and thus cancer-preventive strategies. Future investigations will thus have to be directed toward establishing biomarker panels with methylation thresholds predicting cancer risk in healthy individuals and to evaluate the clinical potential of methylation-suppressive medication.

Numerous studies have shown that aspirin use is associated with a lower incidence of colonic neoplasia, metastatic CRC, and death due to CRC (33–36). Although the genetic makeup of a tumor is likely to play a role in this context (37), our methylation profiling of the normal colonic mucosa adds to the understanding of this cancer protective effect, showing that regular aspirin use stabilizes DNA methylation at promoters of genes controlling critical cancer pathways. This is likely to retard carcinogenesis at different stages, including metastasis, in the colon and other tissues. This insight will thus be instrumental in identifying both patients and healthy individuals who will benefit from aspirin use.

Our study has several strengths. First, we collected normal mucosa samples from an unselected screening cohort, providing for an unbiased analysis of methylation profiles. Second, the age-related and lifestyle-modulated methylation changes observed in the promoters of the two DNA-repair and tumor-suppressor genes *hMLH1* and *MGMT* are an important proof of concept because the pathogenic consequence of their epigenetic silencing is well known. Third, the comparison of our genome-wide methylation data in normal mucosa of a female screening cohort with those of women with CRC enabled us not only to exclude sex-associated methylation variance but also to identify the gene promoters at which age-related hypermethylation is relevant for CRC development.

There are also limitations to our study. Residual confounding by measured or unmeasured additional factors cannot be excluded. To limit biological variation, we investigated only women and, therefore, our findings will have to be validated by independent studies including men. Genome-wide methylation array is largely restricted to CpG islands, thereby limiting the detection of methylation globally.

We conclude that the aging colonic mucosa undergoes changes in gene promoter methylation with rates modulated by lifestyle. The highly variable methylation rates at individual CpGs are consistent with an underlying inherent instability of DNA methylation rather than an outgrowth of subpopulations of tissue cells; a concept supported by many other studies (38–40). We propose that this epigenetic instability contributes to the aging of the colonic epithelium (41), generating opportunities for the evolution of early

cancer-initiating cells (Figure 4C) showing patterns of aberrant DNA methylation typically observed in malignant tissues (2). The finding that lifestyle factors specifically modulate the rate of the underlying DNA methylation change provides a novel paradigm for how the environment modulates cancer risk.

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## Funding

This work was supported by the Swiss Cancer League; Association for International Cancer Research (AICR-11-0247); Stanley Thomas Johnson foundation; and OPO Foundation.

## Notes

We wish to thank Robert Ivanek for critically reviewing the bioinformatics involved in the global methylation analyses, Giancarlo Marra for his contributions to gene expression analyses, and members of the Department of Biomedicine for input and critical discussions throughout the course of the study.

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