Metabolic, hormonal and immunological associations with global DNA methylation among postmenopausal women

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DNA methylation is an epigenetic modification essential for the regulation of gene expression that has been implicated in many diseases, including cancer. Few studies have investigated the wide range of potential predictors of global DNA methylation, including biomarkers. Here, we investigated associations between DNA methylation and dietary factors, sex-steroid hormones, metabolic, lipid, inflammation, immune and one-carbon biomarkers. Data and baseline biomarker measurements were obtained from 173 overweight/obese postmenopausal women. Global DNA methylation in lymphocyte DNA was measured using the pyrosequencing assay for LINE-1 repeats. We used correlations and linear regression analyses to investigate associations between continuous data and DNA methylation, while t-tests were used for categorical data. Secondary analyses stratified by serum folate levels and multivitamin use were also conducted. There was little variability in LINE-1 methylation (66.3–79.5%). Mean LINE-1 methylation was significantly higher among women with elevated glucose levels. Mean LINE-1 methylation was also higher among women with high CD4+/CD8+ ratio, and lower among women with elevated vitamin B6, but neither reached statistical significance. In analyses stratified by folate status, DNA methylation was negatively associated with sex hormone concentrations (estrone, estradiol, testosterone and sex hormone binding globulin) among women with low serum folate levels (n = 53). Conversely, among women with high serum folate levels (n = 53), DNA methylation was positively associated with several immune markers (CD4/CD8 ratio, NK1656/lymphocytes and IgA). Results from this screening suggest that global DNA methylation is generally stable, with differential associations for sex hormones and immune markers depending on one-carbon status.

Introduction

DNA methylation is a reversible epigenetic process of gene regulation and may also impact genomic stability.[1] The mechanism of DNA methylation is particularly important during embryogenesis and carcinogenesis and has been studied mainly in the context of cancer and aging.[1-5] Reduced levels of genomic methylation are commonly observed early during carcinogenesis, and this epigenetic dysregulation, jointly with hypermethylation at specific CpG islands, correlates with disease severity and metastatic capabilities in many tumors.[6,7] There is also evidence that altered DNA methylation patterns are associated with other pathologies such as autoimmune and cardiovascular diseases.[7-9]

Studies of DNA methylation distinguish between hypermethylated sequences in CpG islands near promoter sites of specific genes and global hypomethylation at repetitive sequences as separate mechanisms that foster carcinogenesis.[10,11] Although global hypomethylation is less well understood than CpG island hypermethylation,[12] it has been suggested that a decrease in global DNA methylation may contribute to cancer progression via chromosomal instability and aneuploidy altering chromatin structure.[13-15]

Several studies have revealed the importance of environmental factors in relation to DNA methylation.[16-19] Recent studies have also shown that risk factors across the life course may be associated with adult DNA methylation.[17,20] Nevertheless, the impact of many other lifestyle characteristics on DNA methylation has

Table 1. Baseline characteristics of the study participants

Characteristic	N	Mean ± SD or %
Methylation (%)	173	72.5 ± 2.2
Age (yr)	173	60.6 ± 6.7
Weight (kg)	173	81.6 ± 13.0
Alcohol intake (g/d)	173	4.3 ± 0.6
Body mass index (kg/m²)	173	30.5 ± 3.9
Body fat (%)	173	47.4 ± 4.7
Ethnicity:		
Non-Hispanic white	149	86.0%
African American	7	4.0%
Asian/Pacific Islander	9	5.0%
Hispanic/Latino	2	1.0%
American Indian	2	1.0%
Others	3	2.0%
Ever user of hormones[a]	173	42.2%
Ever smoked > 100 cigarettes[b]	173	46.5%
Ever user of birth control pills	172	61.6%
First-degree relative with cancer	167	59.9%
Multivitamin use (≥ 1 time/week)[c]	111	51.4%

[a]Former users of postmenopausal hormones, as current users were excluded. [b]Former smokers, as current users were excluded. [c]Multivitamin use ≥ 1 time/week during the past 3 mo. Multivitamin use was only assessed among a subset of study participants who participated in the Immune Exercise (IMEX) study

not been well characterized. While some studies have reported associations between age, gender and alcohol and global hypomethylation,[16,20-26] others have not.[11,26-30] Few studies have investigated associations between global DNA methylation and biomarkers, which may represent the systemic hormonal, immunological or inflammatory milieu. In a recent experimental study, an elevated glucose level was observed to increase DNA methylation by enhancing DNA methyltransferase activity.[31] It has been suggested that metabolic control of folate and methyl groups may be under hormonal regulation; however, very little is known about the associations between these hormones and DNA methylation.[32] Folate is a critical source of methyl groups for epigenetic changes and plays an important role in global genomic methylation[12,21,25,33-39] and DNA methylation levels correlate with folate bioavailability.[40]

In this exploratory study, we analyzed dietary, metabolic, lipid, inflammation, immune and sex hormone biomarkers to identify factors that may be associated with DNA methylation at long interspersed nucleotide elements (LINE-1). We also investigated the relationship between LINE-1 methylation and biomarkers of interest stratified by serum folate and multivitamin use, in order to account for one-carbon status. While the goal of this study was to perform an overall screen, we also specifically hypothesized that blood glucose and sex hormone concentrations would be positively associated with DNA methylation. In addition, hypothesized that the associations between dietary, metabolic, lipid, inflammation, immune and sex hormone biomarkers and DNA methylation could depend on one-carbon status; hence, our stratification by serum folate and multivitamin use.

Results

Characteristics of eligible study participants are presented in **Table 1**. The average age of the participants was 60.6 ± 6.7 y and 86% were Caucasian. All participants were overweight or obese, with a mean body mass index (BMI) of 30.5 ± 3.9 kg/m², and a mean percentage body fat of 47.4 ± 4.7%.

There were no associations with DNA methylation in analyses conducted using categorical variables (**Table 2**). Unadjusted t-tests and Pearson's correlations on log-transformed continuous variables in relation to DNA methylation are presented in **Table 3**. There were weak negative correlations between DNA methylation and energy intake, total fat intake and sex hormone binding globulin (SHBG) (r = -0.16 to -0.19) and a weak positive correlation with CD4+/CD8+ ratio (r = 0.18) (**Table 3**). We detected few significant associations with methylation status. DNA methylation was significantly increased among those with higher blood glucose (p = 0.01) and those with a higher CD4+/CD8+ ratio (p = 0.05). A t-test of the median split for DNA methylation with glucose was statistically significant (p = 0.01) with a low mean of 72.0 ± 1.9 (n = 87) and a high mean of 72.9 ± 2.4 (n = 83) for DNA methylation. There was also a significant trend for glucose in quartiles with DNA methylation (p-trend = 0.02) (data not shown). On the other hand, DNA methylation was lower among women with higher dietary vitamin B$_6$ intakes (p = 0.05). There were no significant associations between age, alcohol, BMI, biomarkers of inflammation, sex hormones and methylation status.

Stratification. We stratified our biomarker-methylation analyses by serum folate levels and multivitamin use to investigate associations with biomarkers under a high or low one-carbon status. The associations for sex hormones and immune markers differed substantially by serum folate or multivitamin use (**Table 4 and Fig. 1**). Among women with low serum folate, there were significant inverse correlations between DNA methylation and the following sex hormones: androstenedione (r = -0.27, p = 0.05), estrone (r = -0.30, p = 0.03), estradiol (r = -0.29, p = 0.04), SHBG (r = -0.44, p = 0.001) and testosterone (r = -0.31, p = 0.02). DNA methylation was positively correlated with IgA levels among women with high folate concentrations (r = 0.34, p = 0.01). Among women who did not use multivitamins, there were very similar patterns, with significant negative correlations between DNA methylation and serum estrone, estradiol and SHBG concentrations. Among women who used multivitamins, DNA methylation was negatively correlated with SHBG and % CTL, while being positively correlated with CD4+/CD8+ ratio and IgA levels.

Discussion

This screen of metabolic, immunologic and sex hormone biomarkers associated with global DNA methylation is one of the first comprehensive studies to investigate biologic correlates of DNA methylation. Although there were few associations in the overall analyses (with glucose, vitamin B6 and CD4+/CD8+ ratio), our study revealed many associations between DNA

Table 2. Associations between categorical variables and DNA methylation at baseline

Variable	No		Yes		
	N	DNA methylation	N	DNA methylation	p value
Ever use of postmenopausal hormones[a]	100	72.3 ± 0.2	73	72.5 ± 0.2	0.53
Ever smoke (100+ cigarettes)[b]	92	72.7 ± 0.2	81	72.3 ± 0.3	0.18
Ever use of birth control pills	66	72.5 ± 0.2	106	72.4 ± 0.3	0.71
First-degree relative with cancer	67	72.4 ± 0.2	100	72.4 ± 0.2	0.99
Multivitamin use (≥ 1 time/week)[c]	54	72.4 ± 0.3	57	72.4 ± 0.3	0.91

[a]Former users of postmenopausal hormones, as current users were excluded. [b]Former smokers, as current smokers were excluded. [c]Multivitamin use ≥ 1 time/week during the past 3 mo, assessed among a subset of study participants who participated in the Immune Exercise (IMEX) study.
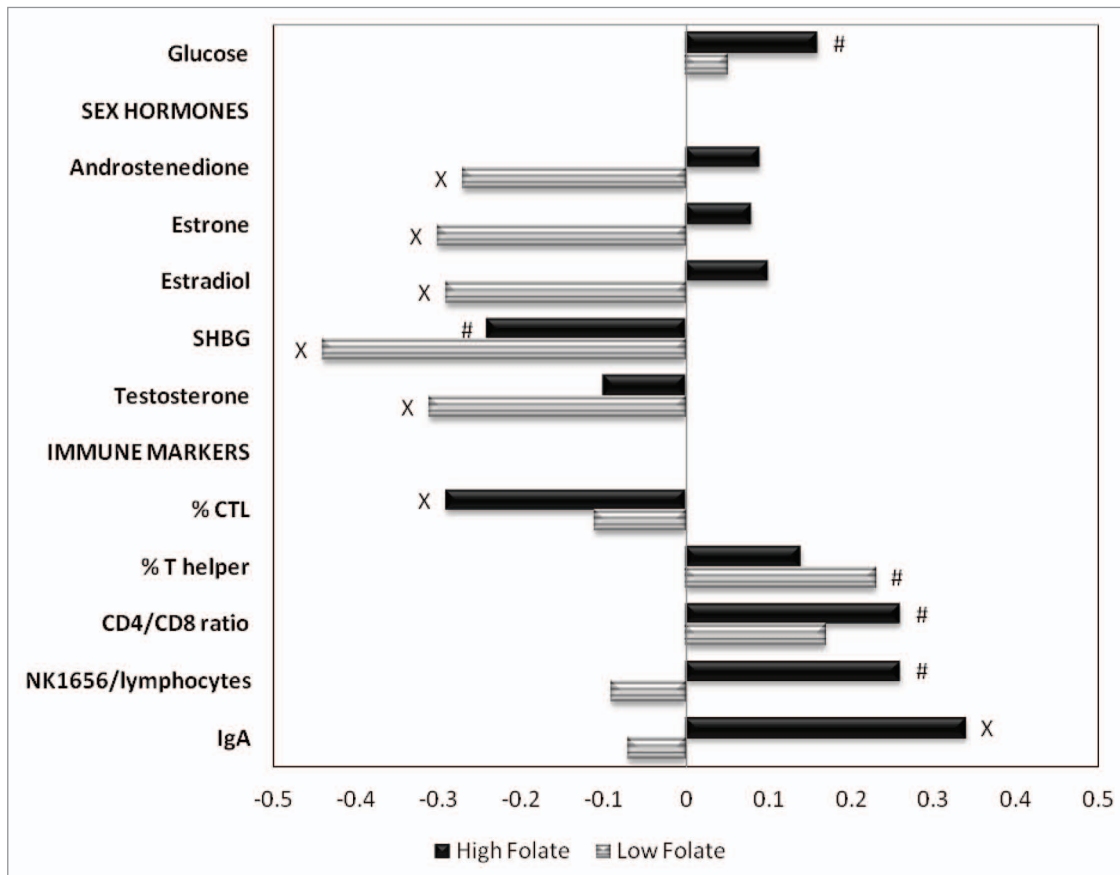
Figure 1. Correlations between markers and DNA methylation, stratified by serum folate concentrations. P values x < 0.05; # ≤ 0.10.

methylation, sex hormones and immune markers when one-carbon status was taken into account.

We observed that individuals with elevated glucose levels had significantly higher mean LINE-1 methylation values. This is similar to what has been recently reported.[41] Pearce and colleagues reported that a 10% increase in LINE-1 methylation was associated with an 0.28 mmol/l increase in fasting glucose concentrations.[41] Furthermore, global DNA methylation has also been shown to be associated with insulin resistance with a 10% increase in global DNA methylation resulting from a 4.55 unit increase in homeostasis model assessment.[42] A potential biological mechanism linking promoter-specific hypermethylation to

glucose levels is that age-related increases in methylation may increase susceptibility to hepatic insulin resistance and diabetes.[43] This hypothesis is supported by evidence from experimental studies, which indicate that high glucose concentration increases promoter-specific methylation of enzymes such as hepatic glucokinase (GCK) and global DNA hypomethylation in hepatic tissue.[31,43]

The effect of age on DNA methylation is controversial. Although the mechanism underlying age-related hypomethylation remains unknown, it has been suggested that repeated rounds of DNA replication may result in progressive loss of methylation in repetitive elements since most normal methylation is

Table 3. Associations between age, biomarkers and DNA methylation

Marker[a]	N	Mean	Standard deviation	p value ≥ vs. < median[b]	Corr r (p)[c]
Age	173	60.6	± 6.7	0.77	0.07 (0.38)
Insulin/IGF/metabolic hormones	**173**				
Glucose (mg/dl)	170	98.0	± 9.1	**0.01**	0.12 (0.11)
Insulin (µg/ml)	173	20.0	± 9.7	0.98	0.03 (0.72)
IGF-1 (ng/ml)	173	107.0	± 36.5	0.84	0.03 (0.74)
IGFBP-3 (µg/ml)	173	4.1	± 1.1	0.64	-0.05 (0.53)
Ghrelin (pg/ml)	173	613.9	± 342.9	0.10	-0.06 (0.43)
Leptin (ng/ml)	173	28.0	± 8.4	0.99	0.02 (0.78)
Lipid metabolism biomarkers	**165**				
Cholesterol (mg/dl)	165	231.6	± 39.6	0.68	0.05 (0.50)
HDL (mg/dl)	165	52.1	± 12.4	0.57	0.01 (0.90)
LDL (mg/dl)	165	152.2	± 38.8	0.65	0.04 (0.61)
VLDL(mg/dl)	165	27.2	± 13.1	0.57	0.01 (0.88)
Triglycerides (mg/dl)	170	135.3	± 65.2	0.53	0.02 (0.85)
One-carbon nutrient biomarkers	**106**				
Serum B12 (pg/ml)	106	390.6	± 189.5	0.85	0.03 (0.79)
Serum folate (ng/ml)	106	56.2	± 34.0	0.66	0.06 (0.52)
Dietary intake	**168**				
Energy (kcal)	168	1643	± 624	0.19	-0.16 **(0.04)**
Alcohol (gm)	168	4.4	± 7.9	0.37	0.08 (0.64)
Total carbohydrates (gm)	168	189.8	± 80.3	0.64	-0.11 (0.14)
Daily fruit (med portions)	168	1.8	± 1.4	0.61	-0.10 (0.22)
Daily vegetables (med portions)	168	2.1	± 1.4	0.23	-0.08 (0.30)
Total fat (gm) BL	168	67.7	± 32.8	0.07	-0.19 **(0.01)**
Folate (µg) BL	168	230.5	± 105.6	0.29	-0.07 (0.35)
Vitamin B6 (mg) BL	168	1.6	± 0.6	**0.05**	-0.10 (0.18)
Vitamin B12 (µg) BL	168	5.3	± 2.6	0.22	-0.08 (0.29)
Body composition	**173**				
BMI (kg/m²)	173	30.5	± 3.9	0.16	0.06 (0.45)
Percent body fat (%)	173	47.4	± 4.7	0.33	0.03 (0.68)
Immune markers	**115**				
% T cells (% of lymphocytes)	112	70.5	± 6.4	0.7	0.05 (0.57)
T cytotoxic cells (% of lymphocytes)	113	20.5	± 8.4	0.51	-0.16 (0.09)
T helper cells (% of lymphocytes)	114	49.3	± 8.1	0.23	0.16 (0.09)
CD4+/CD8+ ratio	113	2.9	± 1.4	**0.05**	**0.18 (0.05)**
NK cells (% lymphocytes)	115	8.6	± 4.3	0.23	0.05 (0.56)
B cells (cells/µl)	115	14.3	± 4.8	0.89	-0.09 (0.33)
IgA (mg/dl)	113	201.5	± 93.4	0.1	0.13 (0.16)
IgG (mg/dl)	114	980.6	± 229.2	0.8	-0.12 (0.20)
IgM (mg/dl)	114	117.6	± 58.2	0.37	-0.13 (0.16)
Biomarkers of inflammation	**115**				
C-Reactive protein (mg/l)	114	0.4	± 0.4	0.48	-0.02 (0.82)
IL-6 (ng/l)	115	3.3	± 3.0	0.91	0.05 (0.60)
Sex hormone biomarkers	**173**				
Estradiol (pg/ml)	173	19.3	± 8.0	0.9	-0.05 (0.56)
Free estradiol (pg/ml)	173	0.5	± 0.2	0.95	0.01 (0.92)

[a]All variables are log transformed. [b]T test compares mean methylation values of groups above and below biomarker median. [c]Correlation coefficients presented are from partial correlations adjusted for age.

Table 3. Associations between age, biomarkers and DNA methylation (continued)

Marker[a]	N	Mean	Standard deviation	p value ≥ vs. < median[b]	Corr r (p)[c]
Bioavailable estradiol (pg/ml)	173	13.4	± 6.0	0.32	0.01 (0.92)
Estrone (pg/ml)	173	46.9	± 17.1	0.76	-0.08 (0.31)
Testosterone (pg/ml)	173	234.5	± 98.9	0.09	-0.07 (0.34)
Free testosterone (pg/ml)	173	5.0	± 2.1	0.73	-0.02 (0.82)
Bioavailable testosterone (pg/ml)	173	12.3	± 5.2	0.83	-0.02 (0.82)
Androstenedione (ng/ml)	173	597.3	± 230.6	0.56	-0.05 (0.51)
DHEA (ng/ml)	173	2.7	± 1.4	0.27	-0.08 (0.31)
DHEA-SO4 (ng/ml)	166	71.7	± 48.4	0.59	-0.07 (0.37)
Prolactin (ng/ml)	172	8.1	± 4.6	0.61	-0.03 (0.67)
Follicle stimulating hormone (mIu/l)	173	66.4	± 23.1	0.09	-0.09 (0.24)
Sex hormone binding globulin (nmol/l)	173	39.5	± 20.8	0.15	-0.16 **(0.04)**

[a]All variables are log transformed. [b]T test compares mean methylation values of groups above and below biomarker median. [c]Correlation coefficients presented are from partial correlations adjusted for age.

in repetitive DNA.[44] Similar to some previous studies,[11,26-30,45-47] we observed no associations between age and DNA methylation. This could be due to the limited age range of the women in our study (55 to 75 y). Bjornsson and colleagues reported time-dependent changes in DNA methylation within the same individual in two longitudinal studies in Iceland and the US.[48] Also, other studies have reported age-associated changes in DNA methylation,[49,50] but these changes have been rather small. More recently, Zhu and colleagues reported that Alu, but not LINE-1, methylation levels decreased with aging, suggesting that changes in DNA methylation due to aging may not be consistent within the genome.[16]

Heavy alcohol intake impairs DNA methylation.[51] Alcohol may potentially stimulate DNA hypomethylation by competing with folate metabolism and decreasing the synthesis of the methyl donor S-adenosylmethionine (SAM).[52] However, similar to our study, most previous studies have not reported any associations between alcohol intake and LINE-1 methylation.[16,29,30,37] This may be because in most of these studies alcohol consumption among study participants was low. For example, in our study, mean alcohol intake was 4.3 ± 0.6 g/d, largely because moderate or heavy alcohol drinkers were excluded from participating in the study. It is likely that the impact of alcohol on DNA methylation is more pronounced among populations with higher alcohol intake.

There are indications that the effects of folate status on DNA methylation are complex, depend on cell type and may be gene and site specific.[53,54] For instance, Slattery and colleagues reported interactions between folate status, polymorphisms in methylenetetrahydrofolate reductase (*MTHFR*) gene and DNA methylation, whereby at low folate levels, individuals who were homozygous for the variant TT genotype of the *MTHFR* gene had DNA hypomethylation.[55] Although the results from our study do not provide evidence for any associations between DNA methylation and serum folate and vitamin B12 levels, our data suggest that one-carbon status may modify the effect of sex hormones and immune markers on global DNA methylation. We observed inverse associations between DNA methylation and

several sex hormones among women with low serum folate levels and those who did not use multivitamins, but not among women with high folate levels and those who used multivitamins. Hence, it may be important for studies investigating the associations between hormones and DNA methylation to examine for possible interactions.

Few studies have investigated the relationship between global DNA methylation and immune function. Based on our stratified analyses, there are suggestions that one-carbon status modifies the associations between immune markers and global DNA methylation. This is because immune markers were associated with global DNA hypomethylation among women with high serum folate and those who use multivitamin but not among women with low serum folate and those not taking multivitamins. T cell DNA demethylation occurs in lupus and rheumatoid arthritis[56] and may be implicated in other immune system disorders. Global age-related hypomethylation occurs in the T-lymphocytes of aging humans,[44,57] although data are sparse on how these age-related methylation changes affect the immune system.[44] The aging process parallels the deterioration in immune function.[14,44] T-cell mediated autoimmune diseases may be included among these age-related diseases that result in changes in the DNA methylation pattern of cells.[14,44] These methylation changes are akin to the changes found in malignant cells, with general genome hypomethylation combined with specific gene hypermethylation and gene silencing.[14]

Gender may be a predictor of LINE-1 methylation, with females having lower methylation levels than males.[23,26,30] It has been suggested that the effect of gender on DNA methylation may be mediated through hormonal differences between males and females, wherein estrogen could be responsible for the lower methylation levels in females and testosterone for the higher methylation levels in males.[26] However, direct evidence linking both hormones to methylation levels is lacking. Similar to the overall observations in our study, El-Maarri and colleagues reported no associations between estrogen and testosterone at LINE-1, as well as Alu repeat regions.[26] Nevertheless, no previous studies have reported on the associations of sex hormones with

Table 4. Univariate associations of biomarkers with DNA methylation, stratified by serum folate or multivitamin use

Marker[a]	Overall[a]	Serum folate[b]		Multivitamin use	
	Corr r(p) (n = 106)	Low corr r (p) (n = 53)	High corr r (p) (n = 53)	No corr r (p) (n = 54)	Yes Corr r (p) (n = 57)
Nutritional biomarkers					
Glucose	0.12 (0.11)	0.05 (0.74)	**0.16 (0.08)**	0.18 (0.20)	0.03 (0.82)
Sex hormones					
Androstenedione	-0.08 (0.38)	**-0.27 (0.05)**	0.09 (0.53)	-0.22 (0.12)	0.02 (0.91)
Estrone	-0.11 (0.28)	**-0.30 (0.03)**	0.08 (0.55)	**-0.36 (0.01)**	0.08 (0.54)
Estradiol	-0.10 (0.31)	**-0.29 (0.04)**	0.10 (0.48)	**-0.34 (0.02)**	0.07 (0.58)
SHBG	**-0.32 (0.00)**	**-0.44 (0.001)**	**-0.24 (0.09)**	**-0.31 (0.03)**	**-0.33 (0.01)**
Testosterone	**-0.21 (0.03)**	**-0.31 (0.02)**	-0.10 (0.46)	**-0.24 (0.09)**	-0.17 (0.20)
Immune markers					
% CTL	**-0.21 (0.03)**	-0.11 (0.50)[d]	**-0.29 (0.04)[d]**	-0.01 (0.92)[e]	**-0.34 (0.01)[f]**
% T helper	**0.18 (0.07)**	**0.23 (0.09)**	0.14 (0.32)[d]	0.22 (0.13)	0.16 (0.22)[f]
CD4/CD8 ratio	**0.22 (0.03)**	0.17 (0.24)[c]	**0.26 (0.06)[d]**	0.10 (0.52)	**0.31 (0.02)[f]**
NK1656/lymphocytes	0.11 (0.27)	-0.09 (0.53)	**0.26 (0.06)**	-0.08 (0.56)	**0.23 (0.08)**
IgA	**0.17 (0.08)**	-0.07 (0.61)[c]	**0.34 (0.01)**	-0.01 (0.93)	**0.38 (0.00)**

All variables are log transformed. P values < 0.10 are marked in bold. [a]Restricted to women with serum folate measurement. [b]Serum folate: low (15.17–49.88 ng/ml), high (50.45–258.71 ng/ml). [c]n = 51; [d]n = 52; [e]n = 53; [f]n = 56.

DNA methylation stratified by serum folate or multivitamin use. In our study, we observed moderate but significant negative associations between sex hormones (estrone, estradiol, androstenedione, testosterone and sex hormone binding globulin) and DNA methylation among women with low serum folate and, with the exception of androstenedione, also among women who did not use multivitamins. Thus, one-carbon status appears to be an important factor in the association between sex hormones and DNA methylation and the associations are only apparent among women who may have lower one-carbon status and thus lower levels of the methyl-donor S-adenosylmethionine.

The strength of our study is that it is one of the first to comprehensively screen metabolic, hormonal and immunological associations with global DNA methylation. The following limitations need to be considered when interpreting the results from our study. Our study was restricted to overweight and obese postmenopausal women; thus, the findings may not be generalizable to other study populations. However, about two-thirds of women in that age group in the US are overweight or obese. Also, the restricted study population may have reduced the variability in methylation values. The LINE-1 pyrosequencing assay used to measure global methylation does not assess site-specific methylation. Furthermore, the relationship between the LINE-1 assay and the methyl group acceptance assay used in previous studies is unknown. The level of LINE-1 methylation is significantly correlated with the amount of genome-wide 5-methyl-cytosine when measured by high performance liquid chromatography.[58] However, this correlation is not perfect and variables such as folate may affect DNA methylation in genomic regions besides LINE-1. Because this was an exploratory study to generate hypotheses for future studies, we did not adjust for multiple comparisons. Hence, with the large number of variables tested and the moderate sample size, some of the variables may have shown statistical significance by chance. Lastly, due to the cross-sectional nature of the study, the associations between the markers and DNA methylation cannot be interpreted as causal.

In this screening study, we noted a limited number of predictors of global DNA methylation differences among overweight/obese postmenopausal women. However, our study revealed that the associations between several markers, particularly sex hormones and immune markers and global DNA methylation are modified by one-carbon status. This knowledge is essential in epidemiological studies investigating the associations of DNA methylation and health. Overall, our exploratory findings await replication in larger studies.

Materials and Methods

Participants. This study was conducted using data from women (n = 173) participating in the Physical Activity for Total Health Study, an exercise trial among overweight and obese sedentary women (age 55 to 75 y) which has been previously described in detail.[59] Participants were eligible if they were not using postmenopausal hormone therapy, sedentary, non-smokers, consumed < 2 alcohol drinks/d, and had no endocrine-related disease or cancer.[59] Women were excluded from the study if they had any factors that might affect intervention adherence or retention or factors that might affect study endpoints (e.g., might affect endogenous hormone levels).[59] As part of the study, several hormonal, metabolic and immunologic biomarkers were assessed. We used baseline data for investigating cross-sectional associations. Informed consent was obtained from all participants and approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board.

Baseline data collection. Height and weight were measured in duplicate. Total body fat was determined using dual-energy

X-ray absorptiometry (Hologic QDR 1500: Hologic Inc.). Information on age, education, income, employment status, marital status, race/ethnicity, use of medications, medical history and smoking history were collected by questionnaire. Measures of dietary and alcohol intake were also evaluated using a 120-item food frequency questionnaire designed and validated at the FHCRC.[60] Use of nutritional supplements was ascertained during an in-person interview and by copying nutritional labels.

Methylation. Buffy coats were assayed for global genomic DNA methylation in lymphocytes using LINE-1 (long interspersed nucleotide elements) bisulfite pyrosequencing. Sodium bisulfite modification of lymphocyte DNA was performed as previously described.[61] Methylation analysis of LINE-1 promoter (GenBank accession number X58075) was investigated using a pyrosequencing-based methylation analysis reported elsewhere.[29]

Metabolic measures. Participants provided a 12-h fasting baseline blood sample, which was processed within 1 h of collection. For all assays, laboratory personnel were blinded to the sample identity. Detailed descriptions on assay methods and reproducibility are given in previous publications.[62-64] Briefly, fasting glucose was quantified on a clinical chemistry autoanalyzer, measuring the combined catalytic activities of hexokinase and glucose-6-phosphate dehydrogenase.[62] Insulin was quantified by a 48-h, polyethylene glycol-accelerated, double-antibody radioimmunoassay.[63] Leptin assays were performed using a commercially available radioimmunoassay (Linco Research).[63] Ghrelin was measured using a modification of a commercial radioimmunoassay (Phoenix Pharmaceuticals).[63] IGF-1 was quantified via a two-site chemiluminescence immunoassay using the Nichols Advantage Specialty System (Nichols Diagnostic Institute).[64] IGFBP-3 was quantified via a competitive protein-binding RIA using the IGFBP-3 100T kit (Nichols Diagnostic Institute)[64].

Lipid metabolism biomarkers. Total cholesterol (TC), high density lipoproteins (HDL) and triglycerides were measured enzymatically using the Hitachi 917 autoanalyzer at the Northwest Lipid Research Laboratories (University of Washington). All lipoprotein assays were performed using previously described methods.[65] We used the Friedewald's equation method[66] to calculate low-density lipoprotein (LDL).

One-carbon nutrient biomarkers. Plasma concentrations of vitamin B12 and total plasma folate were measured in fasting subjects by combined affinity high performance liquid chromatography (HPLC) with electrochemical detection at the Jean Mayer USDA Human Nutrition Aging Center at Tufts University as described previously.[67]

Immune function markers. A subset of Physical Activity for Total Health subjects (n = 114) participated in the Immune Exercise (IMEX) study, for which data on markers of immune function were collected. Methods for measuring immune markers including quality control markers are described elsewhere.[68,69] Briefly, lymphocytes were isolated from whole blood using a whole-blood lysis technique. A four-color flow cytometer (XL-MCL, Beckman Coulter) was used to enumerate subsets of lymphocytes in blood samples. T lymphocyte proliferation studies were conducted using cryo-preserved PBMC, and were performed by 2 methods: tritiated ^{3}H-thymidine incorporation in response to the mitogen phytohemagglutinin (PHA), and by the cell division tracking method in response to anti-CD3. Immunoglobulin A (IgA), G (IgG), and M (IgM) assays were performed by nephelometry using the Behring Nephelometer II analyzer (Dade-Behring Diagnostics).

Sex hormones. Serum hormone assays were performed at the Reproductive Endocrine Research Laboratory (University of Southern California) using methods described in detail elsewhere.[70] Briefly, estradiol, estrone, testosterone, androstenedione, and DHEA were quantified by radioimmunoassay after organic solvent extraction and Celite column partition chromatography.[71,72] Chromatographic separation of the steroids was achieved by use of different concentrations of toluene in isooctane and ethyl acetate in isooctane. Sex hormone-binding globulin (SHBG) was quantified via an immunometric assay, and dehydroepiandrosterone sulfate (DHEAS) was quantified via a competitive immunometric assay (Immulite Analyzer, Diagnostic Products Corp.). Free estradiol and testosterone were calculated using methods described and validated elsewhere.[73-75]

Statistical analysis. The Physical Activity for Total Health study population included 173 women with data on metabolic hormones, lipid metabolism biomarkers, dietary intake, body composition and sex hormones. Missing data for all assays ranged from one to three, except for DHEA-SO$_4$, which was not available for seven women. All biomarkers were log-transformed to reduce departure from normality.

To investigate the associations between biomarkers and DNA methylation, we used Pearson's correlation (because global DNA methylation was nearly normally distributed), t-tests (grouped by approximate median split) were used for categorical data and linear regression analyses were used for continuous data. Methylation values ranged from 66.30% to 79.45%. We investigated unadjusted and age or race/ethnicity-adjusted associations between biomarkers for metabolism, lipid levels, one-carbon nutrients, dietary intake, body composition, immune function and sex hormones with DNA methylation. Because the results were similar for the adjusted and unadjusted analyses, we have presented only results for the unadjusted analyses. All analyses were performed using SAS version 9.1 (SAS Institute). A two-sided p value < 0.05 was considered statistically significant.

We investigated the following categorical variables: regular multivitamin use (yes/no), postmenopausal hormone use (ever/never), oral contraceptive use (ever/never), smoking (ever/never) and a first-degree relative with cancer (yes/no). Glucose was analyzed as an indicator variable for quartiles and as a continuous variable.

We measured associations between the following continuous variables and methylation status: metabolic hormones and lipid metabolism biomarkers, one-carbon nutrient biomarkers, dietary intake, body composition, immune function biomarkers and sex hormones.

Stratification. In secondary analyses, we stratified our investigation of the potential biomarker predictors of DNA methylation by serum folate levels (≤ 50 ng/ml vs. > 50 ng/ml), and

multivitamin use (no vs. yes) (n = 106, available for this analysis). Stratification by these factors was based on the a priori hypothesis that one-carbon status may influence biomarker-methylation relationships. Serum folate values ranged from 15.2–49.9 ng/ml for the low folate group and 50.5–258.7 ng/ml for the high folate group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Ethical Statement

The study was approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board. The study complied with all of the legal requirements.

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