

Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood

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Key words: gender, race/ethnicity, DNA methylation

Abbreviations: LINE-1, interspersed repeat sequences; FFM, fat-free mass; FM, fat mass; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; DFE, dietary folate equivalents

Reduced levels of global DNA methylation are associated with genomic instability and are independent predictors of cancer risk. Little is known about the environmental determinants of global DNA methylation in peripheral blood. We examined the association between demographic and lifestyle factors and levels of global leukocyte DNA methylation in 161 cancer-free subjects enrolled in the North Texas Healthy Heart Study aged 45–75 years in 2008. We used in-person interviews for demographics and lifestyle factors, a self-administrated Block food frequency questionnaire for diet, and bioelectrical impedance analysis and CT-scan for body composition. We measured genomic DNA methylation using bisulfite conversion of DNA and pyrosequencing for LINE-1. Body composition measures including body mass index, waist circumference, areas of subcutaneous fat and visceral fat, percent of fat mass and fat-free mass were not associated with global genomic DNA methylation after controlling the effect of age, gender and race/ethnicity. Instead, female gender was significantly associated with a reduced level of global methylation ($\beta = -2.77$, 95% CI: -4.33, -1.22). Compared to non-Hispanic whites, non-Hispanic blacks ($\beta = -2.02$, 95% CI: -3.55, -0.50) had significantly lower levels of global methylation. No association was found with age, cigarette smoking, alcohol drinking and dietary intake of nutrients in one-carbon metabolism. Global leukocyte DNA methylation differs by gender and race/ethnicity, suggesting these variables need to be taken into consideration in studies of global DNA methylation as an epigenetic marker for cancer.

Introduction

About half of the human genome contains repetitive elements that are intensively methylated and, when hypomethylated, are associated with genomic instability and chromosomal aberrations.¹ Hypomethylation in repetitive elements such as long interspersed repeat sequences (LINE-1) is common in cancer cells and appears to parallel overall genomic hypomethylation.¹ Global leukocyte DNA methylation has been proposed as an epigenetic marker for head and neck squamous cell carcinoma,² bladder cancer,³ breast cancer,⁴ gastric cancer⁵ and colorectal adenoma and cancer^{6,7} even after adjusting for known cancer risk factors.

Few studies have explored the environmental or behavioral determinants of global DNA methylation in humans. Leukocyte DNA methylation differs more in older than in younger monozygotic twins,⁸ and longitudinally, changes over time.⁹ Aging and environmental exposures over the lifetime may therefore

contribute to changes in DNA methylation. Although methylation patterns are known to be tissue specific, recent studies found that age-related methylation showed similar patterns irrespective of tissue type including peripheral blood, suggesting common mechanisms may underlie methylation changes.¹⁰

In this study, we further explore and describe the relation of global leukocyte DNA methylation to aging, demographics and environmental factors in 161 cancer-free individuals aged 45–74 enrolled in the 2008 North Texas Healthy Heart Study.

Results

The mean age of the 161 participants was 57.6 years; 62.7% were women. Non-Hispanic whites, non-Hispanic blacks and Hispanics represented 20.6%, 43.1% and 36.3% of the study population. 39.1% of the participants were overweight and

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Submitted: 01/12/11; Accepted: 02/28/11

DOI: 10.4161/epi.6.5.15335

49.7% were obese. Mean body mass index (BMI) was 31.2 kg/m². 10.6% of the study participants reported current smoking and 32.3% drank alcohol more than 2–4 times per month. Median intake of dietary folate equivalents (DFE) was 402 µg/day. The median level of global DNA methylation, measured as percentage of methylation in LINE-1 repetitive elements in leukocyte DNA, was 73.7%.

Table 1 presents levels of global genomic DNA methylation (%) by personal characteristics and lifestyle factors. Women had a significantly lower level of global genomic DNA methylation than men (73.2 vs. 75.0%, $p < 0.0001$). Non-Hispanic blacks (73.1%) and Hispanics (74.0%) had significantly lower levels of methylation than non-Hispanic whites (75.3%) ($p = 0.001$). Lower levels of global DNA methylation were also found among centrally obese subjects compared to those who were not centrally obese (73.2 vs. 74.7%, $p < 0.01$). No significant differences in global DNA methylation were found for age, education, body mass index, smoking, alcohol drinking and intake of dietary folate equivalents and other one-carbon nutrients. Findings from simple linear regression were consistent with those from analysis of variance (ANOVA). Female gender was negatively associated with global DNA methylation (%) ($\beta = -1.84$, 95% CI: -2.74, -0.94); non-Hispanic blacks ($\beta = -2.25$, 95% CI: -3.43, -1.07) and Hispanics ($\beta = -1.33$, 95% CI: -2.54, -0.11) had significantly lower levels of global DNA methylation compared to non-Hispanic whites; and being centrally obese was also negatively associated with global DNA methylation ($\beta = -1.44$, 95% CI: -2.33, -0.54).

Because levels of global DNA methylation appeared to differ by central obesity, we further examined the association between body composition and global DNA methylation. In a simple linear regression model (model 1), the area of subcutaneous adipose tissue (SAT) (cm²) ($\beta = -0.65$, 95% CI: -1.14, -0.16) but not the area of visceral adipose tissue (VAT), the ratio of subcutaneous adipose tissue to visceral adipose tissue (i.e., the SAT/VAT ratio) ($\beta = -0.70$, 95% CI: -1.19, -0.21), the percent fat mass (FM%) ($\beta = -0.57$, 95% CI: -1.03, -0.11) and the ratio of fat mass to fat-free mass (i.e., the FM/FFM ratio) ($\beta = -0.54$, 95% CI: -1.00, -0.08) were negatively associated with global methylation (Table 2). As expected, the percent fat-free mass (FFM%) was positively correlated with LINE-1 methylation ($\beta = 0.57$, 95% CI: 0.11, 1.03). However, in the multivariate model adjusted for age, gender and race/ethnicity (model 4), the associations between body composition and global methylation were attenuated and became statistically insignificant. Removal of race/ethnicity from model 4 did not change the results (model 3). Removal of gender from model 4, however, resulted in significant associations between body composition and global methylation (model 2), although the associations were weaker than those identified in model 1.

With simultaneous adjustment for age, gender, race/ethnicity, central obesity, SAT/VAT ratio and FM/FFM ratio in the multivariate linear regression model (model 5), the differences in global DNA methylation by gender and race/ethnicity were still present (Table 3). Female gender was negatively associated with global genomic DNA methylation ($\beta = -2.08$, 95% CI: -3.46, -0.71) and non-Hispanic blacks had significantly lower levels of

DNA methylation than non-Hispanic whites ($\beta = -1.91$, 95% CI: -3.22, -0.59). Although Hispanics tended to have lower levels of global DNA methylation than non-Hispanic whites, the difference was statistically insignificant ($\beta = -0.89$, 95% CI: -2.25, 0.47). Age, central obesity, SAT/VAT ratio and FM/FFM ratio were not significantly associated with global DNA methylation. Additional adjustment for other covariates (model 6) yielded similar results (women vs. men: $\beta = -2.77$, 95% CI: -4.33, -1.22; non-Hispanic blacks vs. non-Hispanic whites: $\beta = -2.02$, 95% CI: -3.55, -0.50).

We further assessed whether the significant associations between gender, race/ethnicity and global DNA methylation could be mediated by body composition, dietary folate intake, physical activity, smoking and drinking by comparing the model with age, gender and race/ethnicity to the model additionally adjusted for each of these potential mediators. None of the factors substantially attenuated the effect of gender and race/ethnicity on global DNA methylation (data not shown).

Discussion

In a cancer-free population, we examined the association between age, gender, race/ethnicity, body composition, diet and lifestyle factors and global genomic methylation in leukocyte DNA and found significant differences in global leukocyte DNA methylation by gender and race/ethnicity.

The impact of aging on DNA methylation has been well documented.¹¹ Aging cells show a progressive loss of 5-methylcytosine content.^{12,13} Recent studies have found that CpG islands tended to gain methylation whereas non-island CpGs lose methylation with increasing age.¹⁰ The overall impact of aging on methylation was to reduce the global level of genomic DNA methylation, and this age-related decline in DNA methylation appears to be similar across tissue types.¹⁰ In our study population aged 45–75 years old, the age-related trend in global DNA methylation was in the expected direction but not statistically significant. An age range wider than 45–75 years or a larger sample size may have improved power to detect a significant impact of aging on global DNA methylation.

We found women had a significantly lower level of methylation than men. Although the absolute difference in global genomic DNA methylation is small (1.8%), it is in agreement with three previous studies measuring LINE-1 methylation in peripheral leukocytes. For example, Hsiung et al. reported a 1.17% significantly lower level of LINE-1 methylation in women than in men.² El-Maarri et al. reported the mean differences in LINE-1 methylation by gender ranging between 1.61% and 5.80%: all were significantly lower in women than in men.¹⁴ A combined analysis from five studies by Zhu et al. identified a 0.8% significantly lower level of LINE-1 methylation in women than in men.¹⁵ The reason for this gender specific difference in global DNA methylation is not clear. One of the two chromosomes in women is heavily methylated and transcriptionally inactivated. It has been proposed that X chromosome inactivation may deplete resources required for properly methylating autosomal loci.¹⁴ Lower levels of global methylation in women may also be

Table 1. LINE-1 methylation (%) by demographic and lifestyle factors in the North Texas Healthy Heart study, 2008

		LINE-1 methylation (%)		Linear regression	
		N (%)	Mean (SD)	p value	β (95% CI)
Age (yrs)					
45–55		72 (44.7)	74.2 (3.2)	0.40	-0.42 (-1.46, 0.62)
55–65		54 (33.5)	73.7 (2.7)		
65+		35 (21.7)	73.4 (2.7)		
Gender					
Male		60 (37.3)	75.0 (2.5)	<0.0001	-1.84 (-2.74, -0.94)
Female		101 (62.7)	73.2 (3.0)		
Race/ethnicity					
Non-Hispanic Whites		33 (20.6)	75.3 (3.3)	0.001	-2.25 (-3.43, -1.07)
Non-Hispanic Blacks		69 (43.1)	73.1 (3.0)		
Hispanics		58 (36.5)	74.0 (2.8)		
Education					
High school or less		54 (33.5)	73.9 (2.9)	0.93	-0.04 (-1.01, 0.93)
Some college/college graduate		107 (66.5)	73.8 (2.9)		
Smoking					
Nonsmokers		101 (63.1)	73.7 (3.1)	0.71	-0.06 (-1.58, 1.47)
Former smokers		42 (26.3)	74.2 (2.8)		
Current smokers		17 (10.6)	73.7 (2.7)		
Alcohol					
Never		62 (38.5)	73.5 (2.6)	0.24	0.15 (-0.97, 1.26)
Monthly or less		47 (29.2)	73.7 (2.7)		
≥2–4 times/month		52 (32.3)	74.4 (3.4)		
Body mass index (kg/m²)					
<25		18 (11.2)	74.3 (2.3)	0.43	-0.19 (-1.73, 1.36)
25–30		63 (39.1)	74.1 (2.8)		
≥30		80 (49.7)	73.5 (3.1)		
Central obesity ^a					
No		70 (43.5)	74.7 (2.5)	<0.01	-1.44 (-2.33, -0.54)
Yes		91 (56.5)	73.2 (3.1)		
Dietary folate equivalents (DFE) (μg/1,000 kcal)					
Q1 (<232.7)		35 (25.0)	74.6 (2.7)	0.07	-1.10 (-2.51, 0.32)
Q2 (232.7–273.6)		36 (25.0)	73.9 (2.6)		
Q3 (273.6–336.0)		36 (25.0)	73.9 (2.8)		
Q4 (≥336.0)		36 (25.0)	73.5 (3.8)		
Vitamin B ₁₂ (μg/1,000 kcal)					
Q1 (<1.9)		35 (25.0)	73.2 (2.7)	0.12	1.26 (-0.14, 2.65)
Q2 (1.9–2.4)		36 (25.0)	73.6 (3.8)		
Q3 (2.4–3.1)		36 (25.0)	74.6 (2.7)		
Q4 (≥3.1)		36 (25.0)	74.5 (2.5)		
Vitamin B ₆ (mg/1,000 kcal)					
Q1 (<0.8)		35 (25.0)	74.1 (2.7)	0.13	-0.64 (-2.03, 0.76)
Q2 (0.8–1.0)		36 (25.0)	74.9 (2.6)		
Q3 (1.0–1.2)		36 (25.0)	73.4 (3.9)		
Q4 (≥1.2)		36 (25.0)	73.5 (2.5)		
Riboflavin (mg/1,000 kcal)					

^aCentral obesity is defined as waist circumference >102 cm for men and >88 cm for women.

Table 1. LINE-1 methylation (%) by demographic and lifestyle factors in the North Texas Healthy Heart study, 2008

Q1 (<0.9)	35 (25.0)	73.2 (2.5)		
Q2 (0.9–1.0)	36 (25.0)	73.6 (2.6)		0.43 (-0.97, 1.83)
Q3 (1.0–1.3)	36 (25.0)	74.6 (4.0)		1.36 (-0.04, 2.75)
Q4 (≥1.3)	36 (25.0)	74.4 (2.6)	0.17	1.24 (-0.16, 2.64)
Methionine (mg/1,000 kcal)				
Q1 (<0.7)	35 (25.0)	73.6 (2.9)		
Q2 (0.7–0.8)	36 (25.0)	73.6 (2.9)		-0.06 (-1.47, 1.35)
Q3 (0.8–0.9)	36 (25.0)	74.6 (2.7)		0.97 (-0.44, 2.38)
Q4 (≥0.9)	36 (25.0)	74.1 (2.7)	0.43	0.50 (-0.91, 1.91)

*Central obesity is defined as waist circumference >102 cm for men and >88 cm for women.

due to different levels of dietary folate or other one-carbon nutrients in men and women. In our study population, the median level of dietary folate equivalent was lower in women than in men (397.8 vs. 423.7 $\mu\text{g/day}$); however, the difference was not statistically significant after controlling for the effect of total energy intake ($p = 0.19$). Women may also have a higher folate requirement than men because of regular loss of red blood cells through menstruation but it is unlikely that a higher folate requirement in this population of mostly postmenopausal women can explain the observed gender-specific difference in global methylation. Alcohol drinking and dietary intake of other one-carbon nutrients (vitamins B₂, B₆ and B₁₂ and methionine) did not differ significantly between men and women in this study.

The potential racial/ethnic difference in global DNA methylation has not been widely studied. Hsiung et al. studied 526 healthy individuals who served as controls in a case-control study of head and neck cancer and found a 1.26% significantly higher level of leukocyte LINE-1 methylation in non-Caucasians compared to Caucasians.² In contrast to their findings, we observed a 2.2% lower level of leukocyte LINE-1 methylation in non-Hispanic blacks and 1.3% lower level of leukocyte LINE-1 methylation in Hispanics as compared to non-Hispanic whites although the association for Hispanics did not reach statistical significance. In our study, different racial/ethnic groups had comparable levels of daily intake of dietary folate equivalent and other one-carbon nutrients. Previous studies demonstrated that *MTHFR C677T* polymorphism is more prevalent in Hispanics, compared to non-Hispanics and non-Hispanic blacks carry the lowest frequency of *MTHFR C677T* polymorphism.¹⁶ The *MTHFR C677T* polymorphism is associated with reduced activities of methylenetetrahydrofolate reductase and disruption in DNA methylation. Hispanics may therefore have a lower level of global DNA methylation due to higher frequency of the *MTHFR C677T* polymorphism. However, we observed the lowest levels of global methylation in non-Hispanic blacks despite the fact that non-Hispanics blacks carry the lowest frequency of the *MTHFR C677T* polymorphism. Therefore, the different frequency in *MTHFR C677T* polymorphism across racial/ethnic groups cannot account for observed differences in global DNA methylation in this study. Other genetic polymorphisms of folate-metabolizing enzymes such as *MTHFR A1298C*, *MTRR A66G* and *CBS 844ins68* also vary in minor allele frequencies in

different racial/ethnic groups¹⁷ but their functionality on folate metabolism is less pronounced than that of *MTHFR C677T*. For example, Yang et al. reported that the *MTHFR C677T* polymorphism had a significant effect on serum folate and homocysteine concentrations across all racial/ethnic groups but the impact of other genetic polymorphisms in one-carbon metabolism was heterogeneous in different racial/ethnic groups.¹⁷ The biochemistry of folate metabolism is complex and genetic polymorphisms in folate metabolism may have an interactive effect on global DNA methylation. Some unknown genetic or environmental factors may also contribute to the racial/ethnic difference in global DNA methylation. Since we did not measure genetic polymorphisms in this study, our interpretation is largely speculative. Future studies investigating genetic polymorphisms along with global DNA methylation may help elucidate the difference in global DNA methylation by race/ethnicity.

Few studies have investigated the association between obesity and global leukocyte DNA methylation. In animal models, high-fat diet-induced obesity was found to modify the methylation level of the leptin promoter in adipocytes.¹⁸ Levels of pro-inflammatory cytokines such as IL-6 and TNF α are often elevated in obese individuals. Recent studies also indicated a good correlation between levels of leukocyte DNA methylation and degrees of inflammation, suggesting DNA methylation may represent a novel pathway underlying the association between obesity, inflammation and cancer risk.^{19,20} Although BMI is the most commonly used measure for obesity, it does not adequately capture body fat mass and the distribution of body fat. We characterized body composition using % body fat and areas of subcutaneous fat (SAT) and visceral fat (VAT) (cm²). Interestingly, we found central obesity, subcutaneous fat, SAT/VAT ratio, % fat mass and FM/FFM ratio were significant predictors for low levels of LINE-1 methylation but no associations remained after adjusting for age, gender and race/ethnicity. Gender and race/ethnicity were strongly associated with LINE-1 methylation and appeared to confound the association between body composition and LINE-1 methylation. And gender showed a stronger confounding effect than race/ethnicity. As shown in Table 2, the inclusion of gender in the model substantially attenuated the associations between body composition and methylation and the associations became statistically insignificant. However, the associations were only slightly attenuated and remained statistically significant or

Table 2. Simple and multivariate linear regression of LINE-1 methylation (%) and body composition in the North Texas Healthy Heart study, 2008^a

	Simple linear regression (model 1)	Multivariate linear regression (model 2)	Multivariate linear regression (model 3)	Multivariate linear regression (model 4)
	β (95% CI)	β (95% CI) ^b	β (95% CI) ^c	β (95% CI) ^d
Body mass index (kg/m ²)	-0.12 (-0.58, 0.39)	-0.05 (-0.50, 0.40)	0.05 (-0.40, 0.49)	0.06 (-0.37, 0.50)
Waist circumference (cm)	-0.11 (-0.57, 0.35)	-0.04 (-0.49, 0.41)	-0.22 (-0.66, 0.22)	-0.20 (-0.64, 0.24)
Total adipose tissue (cm ²)	-0.45 (-0.94, 0.05)	-0.37 (-0.87, 0.12)	-0.17 (-0.66, 0.31)	-0.16 (-0.65, 0.32)
Subcutaneous fat (cm ²)	-0.65 (-1.14, -0.16)	-0.49 (-0.99, 0.01)	-0.23 (-0.75, 0.28)	-0.13 (-0.65, 0.38)
Visceral fat (cm ²)	0.20 (-0.30, 0.70)	0.06 (-0.47, 0.58)	-0.02 (-0.50, 0.46)	-0.19 (-0.70, 0.31)
SAT/VAT ratio ^e	-0.70 (-1.19, -0.21)	-0.51 (-1.03, 0.01)	-0.19 (-0.74, 0.36)	0.09 (-0.50, 0.68)
Percent fat mass (%)	-0.57 (-1.03, -0.11)	-0.50 (-0.98, -0.03)	0.11 (-0.50, 0.69)	0.11 (-0.48, 0.70)
Percent fat-free mass (%)	0.57 (0.11, 1.03)	0.50 (0.03, 0.98)	-0.11 (-0.69, 0.48)	-0.11 (-0.70, 0.49)
FM/FFM ratio ^e	-0.54 (-1.00, -0.08)	-0.48 (-0.95, 0.003)	0.15 (-0.43, 0.73)	0.13 (-0.45, 0.72)

^aBody composition measures were standardized to mean of 0 and standard deviation of 1. ^bAdjusted for age and race/ethnicity (non-Hispanic white, non-Hispanic black and Hispanic). ^cAdjusted for age and gender. ^dAdjusted for age, gender and race/ethnicity (non-Hispanic white, non-Hispanic black and Hispanic). ^eSAT/VAT ratio is defined as the ratio of area of subcutaneous adipose tissue (cm²) to the area of visceral adipose tissue (cm²) at the L4-L5 interspaces; FM/FFM ratio is defined as the ratio of fat mass (%FM) to fat-free mass (%FFM).

borderline significant after adjusting for race/ethnicity. Age had a minimal confounding effect of the association between body composition and global DNA methylation. Because the majority of our study population is overweight or obese, we have limited statistical power to detect the impact of obesity on global methylation.

Consistent with previous studies, our study did not support an association between smoking, alcohol drinking and leukocyte DNA methylation.^{2,3,5,15} However, Smith et al. reported that levels of LINE-1 methylation were significantly associated with smoking and alcohol drinking in tissues of squamous cell head and neck cancer.²¹ This suggested that the impact of smoking and drinking on global DNA methylation may be tissue specific. We also did not find significant associations between one-carbon nutrients (e.g., folate, vitamin B₁₂, vitamin B₆, riboflavin and methionine) and global DNA methylation in peripheral blood although we were not able to assess the potential effect modification by *MTHFR* polymorphisms or other genetic polymorphisms involved in one-carbon metabolism. Environmental factors may modify patterns of DNA methylation during a critical window such as embryogenesis or postnatally. For example, a recent study measured global DNA methylation in umbilical cord blood and found a dose-response relationship between prenatal lead exposure and leukocyte DNA methylation.²² Although we did not find significant associations between lifestyle factors and global DNA methylation, the impact of adulthood environmental exposure over the life time cannot be ignored. Future studies with a larger sample size and a longitudinal design are needed for further examining this issue.

It has to be noted that we measured LINE-1 methylation as a surrogate for global DNA methylation by pyrosequencing. Methylation of repetitive elements has been shown to be a major contributor to total genomic DNA methylation in the human genome.²³ Pyrosequencing has been used extensively to measure global DNA methylation and has been shown to be a reproducible assay with a standard deviation of 2%.²³ Although the absolute difference in LINE-1 methylation identified in this study

Table 3. Multivariate linear regression of demographics, body composition and LINE-1 methylation (%) in the North Texas Healthy Heart study, 2008

	Multivariate linear regression (model 5)	Multivariate linear regression (model 6)
	β (95% CI) ^a	β (95% CI) ^b
Age (yrs)		
45–55	ref.	ref.
55–65	-0.59 (-1.65, 0.47)	-0.97 (-2.21, 0.27)
65+	-0.70 (-1.81, 0.52)	-1.16 (-2.52, 0.19)
Gender		
Male	ref.	ref.
Female	-2.08 (-3.46, -0.71)	-2.77 (-4.33, -1.22)
Race/ethnicity		
Non-Hispanic Whites	ref.	ref.
Non-Hispanic Blacks	-1.91 (-3.22, -0.59)	-2.02 (-3.55, -0.50)
Hispanics	-0.89 (-2.25, 0.47)	-1.23 (-3.06, 0.60)
Central obesity^c		
No	ref.	ref.
Yes	-0.85 (-1.94, 0.24)	-1.23 (-3.60, 0.60)
SAT/VAT ratio^c	0.02 (-0.45, 0.49)	0.10 (-0.44, 0.63)
FM/FFM ratio^c	1.20 (-2.52, 4.92)	(-0.58, 8.84)

^aSimultaneously adjusted for each other in the multivariate model.

^bAdditionally adjusted for smoking (nonsmoker, former and current smokers), drinking (never, monthly or less, and ≥ 2 –4 times/month), education (high school or less and some college/college graduate), dietary folate equivalent (quartiles) and daily physical activity (continuous: minutes/day of moderate and vigorous physical activity). ^cCentral obesity is defined as waist circumference >102 cm for men and >88 cm for women; SAT/VAT ratio is defined as the ratio of area of subcutaneous adipose tissue (SAT) to the area of visceral adipose tissue (VAT) at the L4-L5 interspaces; FM/FFM ratio is defined as the ratio of % fat mass (FM) to the % of fat-free mass (FFM).

was small (2–3%), a modest and significant difference in LINE-1 methylation has previously been associated with the risk of head and neck squamous cell carcinoma (74.7% in cases vs. 75.3% in controls),² and in individuals with different exposures to airborne benzene (65.7% in exposed groups vs. 62.3% in unexposed groups).²⁴ These results support the notion that a small difference in global genomic DNA methylation can be etiologically important.

In conclusion, we found significant differences in global genomic DNA methylation in peripheral blood by gender and race/ethnicity in a cancer-free population. The associations are unlikely to be mediated by body composition and other behavioral risk factors such as dietary folate intake, physical activity, smoking and drinking. The biological mechanisms underlying these differences warrant further investigation. Because of the strong association between gender, race/ethnicity and global DNA methylation, confounding and effect modification by these variables needs to be taken into consideration in studies of global DNA methylation as an epigenetic marker for cancer.

Patients and Methods

Study population. The North Texas Healthy Heart Study was initiated in 2006 to assess racial/ethnic differences in cardiovascular serum markers and calcium scores and has been described elsewhere in reference 25–27. Briefly, study participants were recruited from the general population and primary care clinics through the NorTex network (www.hsc.unt.edu/Nortex) in 2006, using either public advertisement or physician's referral. The inclusion criteria include (1) males and females over the age of 45, (2) being Caucasian, Hispanic or African American, (3) no current or previous self-reported history of stroke, peripheral arterial disease, renal failure, heart failure or coronary heart disease and cancer.

161 subjects included in this study are participants who returned for the 2nd study visit in 2008 and completed a survey that elicited information on demographics, smoking, alcohol drinking, medication use, use of oral contraceptives and hormone replacement therapy for women and life stress. Data on physical activity was collected using accelerometers (Actigraph GT1M Monitor). A self-administrated Block food frequency questionnaire (FFQ) was distributed to the study participants asking their usual dietary intake during the last 12 months. A 25 ml sample of whole blood was collected from study participants. Height and weight were measured using standard scales. Waist circumference was measured midway between the bottom of the ribcage and the top of the iliac crest.²⁵ Percent body fat was determined by a Tanita body-fat analyzer using bioelectrical impedance analysis (BIA) (Tanita, Model TBF-300, Arlington Heights, IL). BIA measures the impedance or resistance to a small electrical current as it travels through the body's water pool. The measurements were performed with the participants stepping onto the measuring platform without shoes and after wiping the soles of their feet. Percentage of body fat was estimated using standard prediction equations that incorporate bioelectrical impedance, weight, height, age and sex.²⁸

Abdominal adipose tissue was measured using a 16-slice CT scanner (Toshiba Aquilion 16, Model #TSX-101A, Toshiba America Medical Systems, Tustin, CA). Eight axial CT images of the abdomen were obtained from each subject, with slice number 6 centered on the L4-L5 interspaces. An additional five slices were obtained every 5 cm above the L4-L5 interspaces and two additional slices were obtained 5 and 10 cm below the L4-L5 interspaces. Collimation of 8 mm was used for each axial slice. The cross-sectional areas of total adipose tissue (TAT) (cm²) and visceral adipose tissue (VAT) (cm²) were quantified on each slice using software-derived algorithms (Analyze, version 6.0, Biomedical Imaging Resource, Rochester, MN). Subcutaneous adipose tissue (SAT) (cm²) was calculated from each slice as TAT-VAT. VAT and SAT measured at the L4-L5 interspaces were used as indicators for areas of visceral and subcutaneous adipose tissues for each participant.

Informed consent was obtained from all study participants. The study protocol was approved by the Institutional Review Board of the University of North Texas Health Science Center. Study participants who completed the baseline survey were included in the present study.

Global methylation analysis. Global DNA methylation was measured using bisulfite conversion of DNA and pyrosequencing for LINE-1 in the peripheral blood as described previously by others in reference 24. Briefly, DNAs were bisulfite treated using an EZ DNA Methylation Kit (Zymo Research) following the manufacturer's recommendations. The biotinylated PCR products were purified and pyrosequencing was run on a PyroMark Q24. We used non-CpG cytosine residues as internal controls to verify efficient sodium bisulfite DNA conversion and universal unmethylated and methylated DNAs were run as controls. Methylation quantification was performed using the PyroMark Q24 1.010 software. The degree of methylation was expressed for each DNA locus as percentage methylated cytosine over the sum of methylated and unmethylated cytosine.

Statistical analyses. We compared the level of global DNA methylation by characteristics of the study participants and lifestyle factors using analysis of variance (ANOVA) and linear regression. Global DNA methylation (i.e., LINE-1 methylation) being approximately normally distributed, the continuous LINE-1 methylation % with no transformation was used in linear regression. We defined central obesity as waist circumference >102 cm for men and >88 for women. Density variables (intake/1,000 kcal) were created for one-carbon nutrients (folate, vitamin B₁₂, vitamin B₆, riboflavin and methionine) to adjust for caloric intake,²⁹ which correspond to the effect of increasing the percentage of nutrient intake while keeping total energy intake constant. Because a significant association between central obesity and global genomic DNA methylation was found in simple linear regression, we further examined different measures of body composition and their association with global DNA methylation first in simple linear regression (model 1) and then in multivariate linear regression adjusting for age and gender and race/ethnicity (models 2–4). We defined SAT/VAT ratio as the area of SAT (cm²) divided by the area of VAT (cm²) at the L4-L5 interspaces. We defined FM/FFM ratio as fat mass percentage (FM) divided by fat-free mass

percentage (FFM). Continuous body composition measures were standardized to mean zero and standard deviation one.

We performed multivariate linear regression models (model 5) that simultaneously adjusted for age, gender, race/ethnicity, central obesity, SAT/VAT ratio and FM/FFM ratio in association with global genomic DNA methylation. Last, we generated a fully adjusted linear regression model (model 6) that additionally included education (high school or less and some college/college graduate), smoking (nonsmoker, former and current smokers), drinking (never, monthly or less and ≥ 2 –4 times/month), dietary folate equivalent (quartiles) and daily physical activity

(continuous: minutes/day of moderate and vigorous physical activity). All analyses were performed using SAS (version 9.1; SAS Institute, Cary, NC).

Acknowledgements

This research received supported from University of North Texas School of Public Health and Institute for Cancer Research, and Joe and Jessie Crump Fund for Medical Education. This research was also supported by NIH grant P20MD001633 to Roberto Cardarelli and Joan Carroll, and NIH grant ES009089 to Regina M. Santella. The authors declared no conflicts of interest.

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