

## ORIGINAL ARTICLE

## Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study

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**OBJECTIVE:** Epigenetic mechanisms are increasingly being recognized as an important factor for obesity. The serotonin transporter gene (*SLC6A4*) has a critical role in regulating food intake, body weight and energy balance. This study examines the potential association between *SLC6A4* promoter methylation and obesity measures in a monozygotic (MZ) twin sample.

**METHODS:** We studied 84 MZ twin pairs drawn from the Vietnam Era Twin Registry. Obesity measures include body mass index (BMI), body weight, waist circumference (WC) and waist-hip ratio (WHR). The *SLC6A4* promoter methylation profile in peripheral blood leukocytes was quantified by bisulfite pyrosequencing. The association between methylation variation and obesity parameters was examined by mixed-model regression and matched pair analysis, adjusting for age, smoking, alcohol consumption, physical activity and total daily energy intake. Multiple testing was controlled using the adjusted false discovery rate (*q*-value).

**RESULTS:** Mean methylation level was positively correlated with BMI ( $r=0.29$ ;  $P=0.0002$ ), body weight ( $r=0.31$ ;  $P<0.0001$ ) and WC ( $r=0.20$ ;  $P=0.009$ ), but not WHR. Intra-pair differences in mean methylation were significantly correlated with intra-pair differences in BMI, body weight and WC, but not WHR. On average, a 1% increase in mean methylation was associated with 0.33 kg m<sup>-2</sup> increase in BMI (95% CI: 0.02–0.65;  $P=0.03$ ), 1.16 kg increase in body weight (95% CI, 0.16–2.16;  $P=0.02$ ) and 0.78 cm increase in WC (95% CI, 0.05–1.50;  $P=0.03$ ) after controlling for potential confounders.

**CONCLUSIONS:** *SLC6A4* promoter hypermethylation is significantly associated with an increased prevalence of obesity within a MZ twin study.

*International Journal of Obesity* (2013) 37, 140–145; doi:10.1038/ijo.2012.8; published online 31 January 2012

**Keywords:** DNA methylation; serotonin transporter gene; *SLC6A4*; monozygotic twins

## INTRODUCTION

Obesity has reached epidemic proportions globally in all age and ethnic groups. It is associated with an increased risk of type 2 diabetes, cardiovascular disease and certain types of cancer, posing a substantial public health and economic burden on societies.<sup>1</sup> Despite significant effort, our understanding of the molecular processes underlying obesity remains incomplete. The current obesity epidemic most likely results from the complex interplay between genetic, behavioral and environmental factors. Epigenetic factors act at the interface between the internal genetic landscape and external environmental factors, and thus could be the basis for some specific features of obesity, such as discordance within monozygotic (MZ) twins, and the close relationship with lifestyle factors. Therefore, epigenetic modifications, especially DNA methylation, may have an important role in the pathogenesis of obesity and its associated phenotypes.<sup>2</sup>

Obesity and overweight result from an imbalance between food intake and energy expenditure, leading to storage of excessive calories as body fat. Serotonin (5-hydroxytryptamine, 5-HT) has an important role in the control of energy balance and body weight in the central nervous system.<sup>3</sup> Depletion of central serotonin using selective neurotoxins results in hyperphagia and obesity,<sup>4,5</sup> whereas pharmacological agents that increase serotonin activity in the

central nervous system inhibit food intake and promote weight loss.<sup>6,7</sup> The serotonin transporter is responsible for the reuptake of 5-HT into presynaptic neuron and regulates the extracellular 5-HT concentration, the strength of 5-HT signals and the duration of postsynaptic responses to 5-HT. The serotonin transporter has a pivotal role in serotonin turnover and serotonin level in the synaptic cleft.<sup>8</sup> Consequently, any defects of serotonin transporter may lead to dysregulation of the serotonergic system and could potentially contribute to energy imbalance and influence obesity. Indeed, a promoter gene polymorphism in the serotonin transporter gene has been associated with eating disorder,<sup>9</sup> obesity<sup>10,11</sup> and type 2 diabetes,<sup>12,13</sup> though mixed results exist.<sup>14,15</sup>

The goal of this study was to investigate whether promoter methylation of the serotonin transporter gene (*SLC6A4*) is associated with body mass index (BMI), body weight, waist circumference (WC) and waist-hip ratio (WHR). We tested this hypothesis using a MZ twin sample because epigenetic mechanisms are in part under genetic control.<sup>16</sup> MZ twins are matched on their DNA and many other known and unknown factors, the use of MZ twins could eliminate the confounding by genotype and sex and many other unknown or unmeasured factors, thus providing a useful model for epigenetic research of complex traits such as obesity.

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Received 2 November 2011; revised 15 December 2011; accepted 3 January 2012; published online 31 January 2012

## MATERIALS AND METHODS

### Study population

Twins included in this study were drawn from the Vietnam Era Twin Registry, one of the largest twin registries in the United States.<sup>17</sup> All twins were male veterans who were born between 1946 and 1956. A total of 307 twin pairs (who were raised in the same household) were recruited by the Emory Twin Studies (ETS), which included two companion studies to investigate the role of psychological, behavioral and biological risk factors for subclinical cardiovascular disease in twins. The Emory Twin Studies include male-male twin pairs, including 187 MZ pairs and 120 dizygotic pairs, with an inclusion of two samples of twin pairs discordant for major depression or posttraumatic stress disorder (PTSD). The Emory Twin Studies protocol has been described elsewhere.<sup>18</sup> This research was approved by the Emory Institutional Review Board and all twins signed an informed consent.

The current analysis included 84 MZ twin pairs from the Emory Twin Studies. These twin pairs were selected based on the availability of DNA samples and phenotype data for both members of a twin pair. All twins were examined in pairs at the Emory University General Clinical Research Center between 2002 and 2010, where their medical history was updated. All twins were Caucasians. Zygosity information was determined by DNA analysis.

### Measurement of obesity parameters

Body weight (kilograms) and height (centimeters) were measured when participants wore light clothes and no shoes by trained research staff. BMI was calculated by dividing weight in kilograms by the square of height in meters. Normal body weight, overweight or obesity was defined as  $18.5 < \text{BMI} \leq 24.9 \text{ kg m}^{-2}$ ,  $25.0 < \text{BMI} \leq 29.9 \text{ kg m}^{-2}$  or  $\text{BMI} \geq 30.0 \text{ kg m}^{-2}$ , respectively. WC was measured at the level midway between the lowest rib and the uppermost iliac crest with the subjects standing. Hip circumference was measured at the level of widest circumference over greater trochanters with the legs close together. WHR was calculated as WC divided by hip circumference. All anthropometric measurements were performed twice and the mean value of the two measurements was used for analysis.

### Other measurements

All measurements were performed in the morning after an overnight fast, and both members of a pair were tested at the same time. A medical history and a physical exam were obtained from all twins. Physical activity was assessed by means of a modified version of the Baecke Questionnaire of Habitual Physical Activity used in the Atherosclerosis Risk in Communities Study,<sup>19</sup> a 16-question instrument documenting level of physical activity at work, during sports and non-sports activities. The total physical activity score was used in the analysis. Dietary intake was assessed using the Willett self-administered semi-quantitative food frequency questionnaire,<sup>20</sup> which collected dietary data for the 12-months before testing. Cigarette smoking was classified into current smoker (any number of cigarettes) versus never or past smoker. Pack-years of smoking were calculated as the number of packs of cigarettes smoked per day times the number of years smoked. Information on alcohol consumption was collected by asking about the number of alcoholic drinks (beer, wine or liquor) consumed in a typical week. The total amount of alcohol consumption (in grams) per week was estimated based on the following algorithms: 4 oz of wine contains 10.8 g, 12 oz of beer contains 13.2 g and 1.5 oz of liquor contains 15.1 g of ethanol.

### DNA methylation analyses by quantitative bisulfite pyrosequencing

Promoter methylation level of the serotonin transporter gene (*SLC6A4*) in peripheral blood leukocytes was determined using quantitative bisulfite pyrosequencing by the EpigenDx Inc. (Worcester, MA, USA). Briefly, we assayed 20 CpG dinucleotides in the promoter region of the *SLC6A4* from -209 to -67 bps from the transcriptional start site, based on Ensembl Gene ID ENSG00000108576 and the Transcript ID ENST00000394821. The *SLC6A4* assay was designed to capture the CpG sites in the promoter region immediately upstream of the transcriptional start site. The assay is targeted to the antisense sequence of *SLC6A4* gene. To sequence these selected

**Table 1.** Targeted regions of *SLC6A4* by pyrosequencing

Genomic DNA target sequence	5'-CGCCGCCAAAGAGCTCTGAAGAATTTT GCG-3' 5'-TCACCTTGAGGCGAATAAACTTAATGCTT CCC-3'
Bisulfite converted target sequence	5'-YGTGYTTAAAGAGTTTTGAAGAATTTT GYGTTA-3' 5'-TTTTGAGGYGAATAAAATTTAATGTTTTT-3'
Pyrosequencing assay (dispensation order)	5'-GTCTGTCGCTAGAGTTGAGATTAGTCGTAT TGATGTCGAT-3'
PCR product length (bp)	205

CpG sites, we designed pyrosequencing assays and tested for PCR preferential amplification and quantitative pyrosequencing analysis. The target sequences (genomic DNA and bisulfite converted DNA) from the pyrosequencing assays are listed in Table 1. Table 2 presents the genomic location of the targeted CpG sites (with respect to transcriptional start site) by pyrosequencing.

The bisulfite conversion was performed with 500 ng genomic DNA isolated from peripheral blood leukocytes using the EZ DNA methylation kit (ZymoResearch, Inc., Irvine, CA, USA). The PCR reaction was performed with 0.2  $\mu\text{M}$  of each primer with one of the PCR primers being biotinylated in order to purify the final PCR product using sepharose beads. The PCR product was bound to streptavidin sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the sepharose beads containing the immobilized PCR product were purified, washed and denatured using 0.2 M NaOH solution and rewashed using the pyrosequencing Vacuum Prep Tool (Qiagen Pyrosequencing, Valencia, CA, USA) as recommended by the manufacturer. Then 0.5  $\mu\text{M}$  pyrosequencing primer was annealed to the purified single-stranded PCR product. A total of 10  $\mu\text{l}$  of the PCR products were sequenced by pyrosequencing PSQ96 HS System (Qiagen Pyrosequencing) following the manufacturer's instructions (Qiagen Pyrosequencing). The methylation status of each CpG site was analyzed individually as an artificial T/C SNP using QCpG software (Qiagen Pyrosequencing). The methylation level at each CpG site for each sample was calculated as the percentage of the methylated alleles over the sum of methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites within the targeted region. Each pyrosequencing assay was done on duplicate samples, and each pyrosequencing assay was performed a minimum of two times. For quality control, each experiment included non-CpG cytosines as internal controls to verify efficient sodium bisulfite DNA conversion. We also included unmethylated and methylated DNAs as controls in each run. In addition, we performed PCR bias testing using pyrosequencing by mixing the unmethylated DNA control and *in vitro* methylated DNA at different ratios (0, 20, 40 up to 100%) followed by bisulfite modification, PCR and pyrosequencing analysis. The percent methylation obtained from the mixing study showed high correlation with expected methylation percentages ( $r^2 \geq 0.97$ ), indicating high-quality methylation data.

### Statistical analyses

Because methylation data at some CpG sites are not normally distributed, we performed nonparametric statistical analysis. To adjust for multiple testing, we used the Benjamini-Hochberg false discovery rate procedure<sup>21</sup> to correct for the number of CpG sites evaluated. An false discovery rate-adjusted *P*-value (*q*-value) threshold of 0.05 was used to determine significance. The PROC MULTTEST procedure in SAS 9.2 was used to calculate the *q*-value. The following statistical analyses were performed to examine the association between *SLC6A4* promoter methylation level and obesity:

*Regression analyses by treating twins as individuals:* We examined whether DNA methylation variation was associated with obesity measures (BMI, body weight, WC and WHR), adjusting for age, smoking and alcohol consumption (g per week). These analyses were done using mixed modeling, in which twin pair was included as random effect to account for the within twin pair correlations.

**Table 2.** *SLC6A4* promoter CpG sites examined by pyrosequencing

Targeted CpG sites	Distance relative to TSS (bp)	Genomic position (GCRh37/hg19)
1	-69	Chr17: 28562784
2	-72	Chr17: 28562787
3	-99	Chr17: 28562814
4	-112	Chr17: 28562827
5	-133	Chr17: 28562848
6	-135	Chr17: 28562850
7	-139	Chr17: 28562854
8	-141	Chr17: 28562856
9	-147	Chr17: 28562862
10	-149	Chr17: 28562864
11	-155	Chr17: 28562870
12	-170	Chr17: 28562885
13	-174	Chr17: 28562889
14	-188	Chr17: 28562903
15	-190	Chr17: 28562905
16	-195	Chr17: 28562910
17	-200	Chr17: 28562915
18	-207	Chr17: 28562922
19	-209	Chr17: 28562924
20	-213	Chr17: 28562928

Abbreviation: Chr, chromosome.

*Matched pair analyses by considering twins as members of a twin pair:* We first calculated intra-pair difference in methylation level within a twin pair, defined as either the absolute difference or the actual difference in methylation level between two members of a twin pair. The intra-pair differences in obese measures and other continuous variables were similarly calculated. Spearman's rank correlations between intra-pair difference in each of the obese measures, separately, and intra-pair difference in DNA methylation level at each CpG site were then calculated. In addition, we conducted robust regression by regressing the intra-pair difference in obesity parameter (dependent variable) on the intra-pair difference in DNA methylation level (independent variable) at each CpG site, adjusting for intra-pair differences in smoking (pack-year) and alcohol consumption (g per week) between two members of a twin pair.

*Sensitivity analyses:* As described before, our study included a random sample of twins with depression or PTSD. To examine whether depression (as measured by BDI scores) or PTSD potentially confounds the results, we conducted sensitivity analyses by further adjusting for depressive symptoms or PTSD ( $n=35$ , including 9 pairs and 17 singletons). Moreover, we performed statistical analyses to examine the potential impact of physical activity and diet on our results by further adjusting for physical activity level and total daily energy intake (Kcal per day) in the above statistical models.

## RESULTS

Table 3 presents the demographic characteristics of the twins included in this analysis. The age of the twins ranged from 48 to 61 years with a mean of 55. About 41% and 44% of the twin participants are obese and overweight, respectively. Except for body height, all other obese measures (BMI, body weight, WC and WHR) are highly correlated (all  $P$ 's  $<0.0001$ ). Twins included in the current analysis were not different from those not included in terms of obese measures (e.g., BMI, body weight, WC and WHR) and other covariates (e.g., pack-year, alcohol consumption, physical activity level, total energy intake and BDI scores).

Among the 20 examined CpG sites, methylation levels at 19 sites were highly correlated (all  $P$ 's  $<0.0001$ ). Methylation level of one CpG site (position 3) was significantly correlated ( $r=0.72$ ;  $P<0.0001$ ) with that of another site (position 4), but not others. Mean methylation level across the 20 promoter CpG sites was

**Table 3.** Demographic, clinical and laboratory characteristics of the twins

Characteristic	Mean $\pm$ s.d. or %
Age (years)	55.1 $\pm$ 2.8
Type 2 diabetes (%)	11.4
Hypertension (%)	36.5
Current smoking (pack-year)	22.9 $\pm$ 24.1
Alcohol consumption (g per week)	12.5 $\pm$ 36.3
Overweight (%)	43.6
Obesity (%)	40.6
Body weight (kg)	90.4 $\pm$ 15.9
Body height (cm)	175.4 $\pm$ 6.5
Body mass index (kg m <sup>-2</sup> )	29.4 $\pm$ 4.8
Waist circumference (cm)	99.3 $\pm$ 12.5
Hips circumference (cm)	104.4 $\pm$ 9.3
Waist-to-hip ratio	0.95 $\pm$ 0.06
Systolic blood pressure (mm Hg)	129.3 $\pm$ 17.5
Diastolic blood pressure (mm Hg)	81.4 $\pm$ 11.6
High-density lipoprotein (mg dl <sup>-1</sup> )	37.6 $\pm$ 10.7
Low-density lipoprotein (mg dl <sup>-1</sup> )	123.3 $\pm$ 36.8
Total triglyceride (mg dl <sup>-1</sup> )	177.5 $\pm$ 102.5
Mean <i>SLC6A4</i> methylation level (%)	11.0 $\pm$ 4.2

11.0%, with the highest and lowest being 16.7% and 3.0%, respectively. Mean methylation levels in normal, overweight and obese twins were 10.1%, 11.1% and 11.4%, respectively. Mean methylation level was positively correlated with BMI ( $r=0.29$ ;  $P=0.0002$ ), body weight ( $r=0.31$ ;  $P<0.0001$ ) and WC ( $r=0.20$ ;  $P=0.009$ ), but not body height ( $r=0.05$ ;  $P=0.48$ ) and WHR ( $r=0.06$ ;  $P=0.41$ ). DNA methylation levels of the two members within a pair were highly correlated at all studied CpG sites (all  $P$ 's  $<0.0001$ ). The mean DNA methylation level of the two twins within a pair was also significantly correlated ( $r=0.41$ ,  $P=0.0001$ ).

Mixed model regression analyses by treating twins as individuals demonstrate that methylation levels at most of the examined CpG sites were significantly associated with BMI, body weight and WC, but not body height and WHR, after adjusting for age, smoking and alcohol consumption. Mean methylation level of the examined CpG sites across the promoter region of *SLC6A4* was also significantly associated with these three obesity parameters (Table 4). On average, a 1% increase in DNA methylation was associated with 0.33 kg m<sup>-2</sup> increase in BMI (95% CI, 0.02–0.65;  $P=0.03$ ), 1.16 kg increase in body weight (95% CI, 0.16–2.16;  $P=0.02$ ) and 0.78 cm increase in WC (95% CI, 0.05–1.50;  $P=0.03$ ).

Results for the association between *SLC6A4* methylation and depressive symptoms using absolute and actual intra-pair differences are very close. Recognizing that twin 1 and twin 2 in a pair were allocated randomly, here we choose to present the results by absolute difference. Intra-pair differences in DNA methylation level were significantly correlated with intra-pair differences in BMI and body weight (CpG sites 12, 13, 15, 16 and 20; all  $r \geq 0.24$ , all  $P$ 's  $\leq 0.04$ ) as well as WC (CpG sites 1, 3, 7, 10 and 12; all  $r \geq 0.22$ , all  $P$ 's  $\leq 0.05$ ). However, there was no correlation between intra-pair difference in DNA methylation and intra-pair difference in body height and WHR.

Results for regression analyses using intra-pair differences are shown in Table 5. It shows that intra-pair differences in DNA methylation level were significantly associated with intra-pair differences in BMI and body weight (a total of nine CpG sites out of the twenty studied CpG sites) as well as WC (a total of eight CpG sites out of the twenty examined CpG sites), after adjusting for smoking and alcohol consumption. On average, a 1% increase in the difference in mean DNA methylation level was associated with 0.12 kg m<sup>-2</sup> increase in the difference in BMI (95% CI: 0.01–0.22;

**Table 4.** Association between *SLC6A4* promoter methylation and obesity measures by mixed model

CpG site	BMI (kg m <sup>-2</sup> ) (95% CI; P)	BW (kg) (95% CI; P)	WC (cm) (95% CI; P)
<i>Position</i>			
1	0.43 (-0.08 to 0.94; 0.10)	1.53 (-0.02 to 3.08; 0.05)	1.01 (-0.01 to 2.03; 0.05)
2	0.12 (-0.39 to 0.62; 0.65)	0.49 (-1.28 to 2.27; 0.59)	0.27 (-0.85 to 1.38; 0.64)
3	0.06 (-0.08 to 0.19; 0.39)	0.08 (-0.37 to 0.53; 0.72)	0.01 (-0.27 to 0.27; 0.97)
4	0.05 (-0.02 to 0.13; 0.16)	0.08 (-0.18 to 0.34; 0.56)	0.06 (-0.11 to 0.22; 0.49)
5	<b>0.29 (0.05 to 0.54; 0.01)<sup>a</sup></b>	<b>1.03 (0.28 to 1.78; 0.007)<sup>a</sup></b>	<b>0.69 (0.12 to 0.25; 0.01)<sup>a</sup></b>
6	<b>0.37 (0.07 to 0.67; 0.01)<sup>a</sup></b>	<b>1.27 (0.31 to 2.23; 0.007)<sup>a</sup></b>	<b>0.86 (0.17 to 1.55; 0.01)<sup>a</sup></b>
7	<b>0.26 (0.03 to 0.55; 0.01)<sup>a</sup></b>	<b>0.88 (0.01 to 1.34; 0.03)<sup>a</sup></b>	<b>0.62 (0.01 to 1.28; 0.03)<sup>a</sup></b>
8	<b>0.41 (0.03 to 0.79; 0.03)<sup>a</sup></b>	<b>1.39 (0.18 to 2.60; 0.02)<sup>a</sup></b>	<b>0.94 (0.05 to 1.83; 0.03)<sup>a</sup></b>
9	0.17 (-0.03 to 0.37; 0.09)	0.62 (-0.02 to 1.25; 0.05)	0.43 (-0.03 to 0.89; 0.06)
10	<b>0.31 (0.02 to 0.39; 0.03)<sup>a</sup></b>	<b>1.06 (0.06 to 2.06; 0.03)<sup>a</sup></b>	<b>0.70 (0.01 to 1.40; 0.04)<sup>a</sup></b>
11	<b>0.24 (0.01 to 0.47; 0.03)<sup>a</sup></b>	<b>0.85 (0.13 to 1.56; 0.02)<sup>a</sup></b>	<b>0.54 (0.01 to 1.07; 0.04)<sup>a</sup></b>
12	<b>0.26 (0.02 to 0.45; 0.03)<sup>a</sup></b>	<b>0.88 (0.05 to 1.35; 0.02)<sup>a</sup></b>	<b>0.64 (0.03 to 1.29; 0.03)<sup>a</sup></b>
13	<b>0.19 (0.01 to 0.38; 0.04)<sup>a</sup></b>	<b>0.68 (0.06 to 1.29; 0.03)<sup>a</sup></b>	<b>0.45 (0.01 to 0.91; 0.02)<sup>a</sup></b>
14	<b>0.19 (0.01 to 0.36; 0.03)<sup>a</sup></b>	<b>0.65 (0.09 to 1.19; 0.02)<sup>a</sup></b>	<b>0.44 (0.03 to 0.85; 0.02)<sup>a</sup></b>
15	<b>0.31 (0.02 to 0.60; 0.03)<sup>a</sup></b>	<b>1.10 (0.17 to 2.03; 0.02)<sup>a</sup></b>	<b>0.73 (0.06 to 1.39; 0.03)<sup>a</sup></b>
16	<b>0.22 (0.02 to 0.44; 0.02)<sup>a</sup></b>	<b>0.81 (0.11 to 1.50; 0.02)<sup>a</sup></b>	<b>0.53 (0.03 to 1.03; 0.03)<sup>a</sup></b>
17	0.25 (-0.04 to 0.54; 0.08)	<b>0.94 (0.03 to 1.85; 0.04)<sup>a</sup></b>	0.65 (-0.01 to 1.30; 0.05)
18	<b>0.26 (0.03 to 0.50; 0.03)<sup>a</sup></b>	<b>0.91 (0.01 to 1.61; 0.03)<sup>a</sup></b>	0.63 (-0.02 to 1.27; 0.06)
19	<b>0.41 (0.05 to 0.77; 0.02)<sup>a</sup></b>	<b>0.15 (0.36 to 2.62; 0.01)<sup>a</sup></b>	<b>0.97 (0.11 to 1.82; 0.02)<sup>a</sup></b>
20	<b>0.20 (0.02 to 0.38; 0.03)<sup>a</sup></b>	<b>0.73 (0.14 to 1.31; 0.01)<sup>a</sup></b>	<b>0.46 (0.04 to 0.88; 0.03)<sup>a</sup></b>
Mean	<b>0.33 (0.02 to 0.65; 0.03)<sup>a</sup></b>	<b>1.16 (0.16 to 2.16; 0.02)<sup>a</sup></b>	<b>0.78 (0.05 to 1.50; 0.03)<sup>a</sup></b>

Abbreviations: BMI, body mass index; BW, body weight; WC, waist circumference. All *P*-values adjusted for age, smoking and alcohol consumption. Those highlighted in bold indicate that the associations remained statistically significant after further adjusting for physical activity, total daily energy intake and depressive symptoms. <sup>a</sup>*P*-values remained significant after correction for multiple testing by adjusted-FDR (*q*-value) at the 0.05 level.

**Table 5.** Change in the difference in obese measures per 1% increase in the difference in *SLC6A4* methylation level between two members of a twin pair by robust regression

CpG site	Difference in BMI (95% CI; P)	Difference in BW (95% CI; P)	Difference in WC (95% CI; P)
<i>Position</i>			
1	0.16 (-0.02 to 0.26; 0.11)	0.63 (-0.37 to 1.62; 0.22)	0.39 (-0.54 to 1.32; 0.41)
2	0.26 (-0.15 to 0.67; 0.21)	1.01 (-0.14 to 2.16; 0.08)	0.46 (-0.62 to 1.53; 0.41)
3	0.04 (-0.22 to 0.31; 0.75)	0.14 (-0.65 to 0.93; 0.72)	0.37 (-0.34 to 1.07; 0.31)
4	0.03 (-0.11 to 0.05; 0.49)	0.04 (-0.28 to 0.19; 0.72)	0.08 (-0.13 to 0.29; 0.45)
5	<b>0.16 (0.05 to 0.28; 0.007)<sup>a</sup></b>	<b>0.57 (0.24 to 0.91; 0.0009)<sup>a</sup></b>	<b>0.37 (0.04 to 0.69; 0.02)<sup>a</sup></b>
6	0.13 (-0.03 to 0.28; 0.11)	0.41 (-0.05 to 0.88; 0.08)	0.39 (-0.02 to 0.81; 0.06)
7	0.06 (-0.04 to 0.16; 0.26)	0.22 (-0.08 to 0.53; 0.16)	0.27 (-0.02 to 0.55; 0.07)
8	<b>0.16 (0.004 to 0.27; 0.05)</b>	<b>0.65 (0.10 to 1.20; 0.02)<sup>a</sup></b>	<b>0.56 (0.04 to 1.08; 0.03)<sup>a</sup></b>
9	0.06 (-0.01 to 0.12; 0.11)	0.21 (-0.01 to 0.43; 0.06)	0.20 (0.002 to 0.34; 0.05)
10	0.08 (-0.08 to 0.25; 0.32)	0.30 (-0.22 to 0.82; 0.26)	0.23 (-0.23 to 0.70; 0.33)
11	0.07 (-0.03 to 0.16; 0.16)	0.26 (-0.03 to 0.56; 0.07)	0.20 (-0.06 to 0.46; 0.14)
12	<b>0.12 (0.01 to 0.25; 0.05)</b>	<b>0.44 (0.06 to 0.82; 0.02)<sup>a</sup></b>	<b>0.31 (0.04 to 0.63; 0.02)<sup>a</sup></b>
13	<b>0.09 (0.006 to 0.16; 0.03)<sup>a</sup></b>	<b>0.31 (0.05 to 0.54; 0.02)<sup>a</sup></b>	<b>0.20 (0.005 to 0.41; 0.05)</b>
14	<b>0.08 (0.008 to 0.15; 0.03)<sup>a</sup></b>	<b>0.26 (0.04 to 0.48; 0.02)<sup>a</sup></b>	<b>0.21 (0.004 to 0.42; 0.03)<sup>a</sup></b>
15	<b>0.14 (0.002 to 0.27; 0.04)</b>	<b>0.43 (0.002 to 0.88; 0.05)</b>	<b>0.28 (0.004 to 0.71; 0.05)</b>
16	<b>0.12 (0.01 to 0.23; 0.03)<sup>a</sup></b>	<b>0.45 (0.10 to 0.79; 0.01)<sup>a</sup></b>	0.20 (-0.13 to 0.52; 0.24)
17	0.11 (-0.02 to 0.26; 0.08)	0.39 (-0.002 to 0.79; 0.05)	0.22 (-0.14 to 0.59; 0.24)
18	0.08 (-0.04 to 0.22; 0.19)	0.28 (-0.10 to 0.68; 0.15)	0.26 (-0.08 to 0.60; 0.14)
19	<b>0.20 (0.04 to 0.36; 0.01)<sup>a</sup></b>	<b>0.63 (0.15 to 1.11; 0.009)<sup>a</sup></b>	<b>0.60 (0.12 to 1.08; 0.01)<sup>a</sup></b>
20	<b>0.07 (0.01 to 0.14; 0.05)</b>	<b>0.27 (0.01 to 0.53; 0.04)</b>	<b>0.20 (0.01 to 0.42; 0.05)</b>
Mean	<b>0.12 (0.01 to 0.22; 0.03)<sup>a</sup></b>	<b>0.41 (0.01 to 0.81; 0.03)<sup>a</sup></b>	<b>0.35 (0.002 to 0.48; 0.04)</b>

Abbreviations: BMI, body mass index; BW, body weight; WC, waist circumference. All *P*-values adjusted for differences in pack-year and alcohol consumption. Those highlighted in bold indicate that the associations remained statistically significant after further adjusting for physical activity, total daily energy intake and depressive symptoms. <sup>a</sup>*P*-values remained significant after correction for multiple testing by adjusted-FDR (*q*-value) at the 0.05 level.

*P* = 0.03), 0.41 kg increase in the difference in body weight (95% CI, 0.01–0.81; *P* = 0.03) and 0.35 cm increase in the difference in WC (95% CI, 0.002–0.48; *P* = 0.04). However, intra-pair differences in DNA methylation level were not associated with intra-pair differences in body height and WHR. Further adjustments for physical activity, total energy intake and depressive symptoms/PTSD slightly attenuated the associations but results remained statistically significant.

## DISCUSSION

We demonstrate that promoter methylation of the serotonin transporter gene in peripheral blood leukocytes were significantly associated with obesity parameters. Specifically, *SLC6A4* promoter hypermethylation was significantly associated with an increase in obesity measures (e.g., BMI, body weight or WC). The serotonergic system is involved in the complex behavioral, psychological and physiological processes in maintaining energy balance.<sup>22,23</sup>

A sufficient amount of serotonin in the central nervous system is necessary for the normal functioning of these biological activities. Animal studies have shown that the loss of brain serotonin was associated with overeating and increased body weight,<sup>5</sup> and serotonin transporter deficiency increases abdominal fat.<sup>24</sup> Moreover, serotonin agonists or serotonin reuptake inhibitors decreases food intake and induces weight loss in rodents.<sup>25</sup> Studies in humans have also demonstrated that abnormal hypothalamic serotonergic neurotransmission is associated with eating disturbances.<sup>6,26</sup> In this context, our finding about the association between higher DNA methylation level in the serotonin transporter gene promoter region and an increased risk for obesity is in agreement with previous literatures.

DNA methylation usually inhibits or silences gene expression.<sup>27</sup> Hypermethylation in the serotonin transporter gene promoter region could lead to reduced or silenced gene expression, which in turn leads to low-serotonin uptake and a deficiency of serotonin activity. Indeed, evidence of serotonin deficiency has been reported in obese individuals.<sup>28</sup> Alternatively, given the critical role of serotonergic system in stress responses, it is also possible that hypermethylation-induced serotonin deficiency could result in abnormal responses to stress, a mechanism known to be involved in the pathogenesis of obesity.<sup>29</sup>

DNA methylation is site specific. We therefore presented the association of methylation variation at each individual CpG site as well as their mean with obesity measures. As shown in Table 4, it appears that there exist three epigenetic domains (–133 to –141bp, –149 to –195bp and –207 to –213bp), each of which exhibits increased methylation variation that might be associated with obesity. These differentially methylated regions are also observed in cancers.<sup>30</sup>

BMI is a convenient surrogate measure of total fat mass.<sup>31</sup> It offers a straightforward, inexpensive and useful measure for overweight and obesity and is typically used as an indicator for general obesity. Here we found that aberrant promoter methylation of the serotonin transporter gene was significantly associated with BMI and body weight. However, *SLC6A4* methylation variation was not associated with body height, suggesting that the observed association between *SLC6A4* methylation and BMI is primarily driven by body weight instead of body height.

WC and WHR are commonly used anthropometric measures for adiposity. Although WC has been consistently correlated with the proportion of abdominal fat,<sup>32</sup> the relationship between WHR and abdominal adiposity was mixed in previous studies.<sup>33–35</sup> In this analysis, we identified significant correlation between *SLC6A4* promoter methylation variation and WC, but no correlation was observed between DNA methylation and WHR. This could be because of either a relatively large waist or a small hip girth, or *vice versa*, which leads to an uninterpretable or misleading WHR in relation to the amount of a person's abdominal fat. Our findings are in line with previous studies, indicating that WHR is less useful in reflecting the accumulation of abdominal adiposity. WC may be a better indicator for abdominal adiposity than WHR.

Our study has a few limitations. First, because of practical difficulties in obtaining tissues from living individuals, methylation levels were tested in peripheral blood leukocytes, but not directly from the primary affected organs in obesity, for example, adipocytes. Therefore, our results may not provide a direct index of DNA methylation in the system of adipose metabolism. Second, our epigenetic data were collected from DNA derived from whole blood leukocytes, which is a mixture of many cell types; as such, we were unable to assess blood cell-specific differences in methylation changes. Third, DNA methylation influences disease risk through regulating gene expression, which was unable to be evaluated in this study because of the lack of gene expression data from fresh leukocytes or adipose tissues. Fourth, though BMI

and WC are useful and reliable measures for total body fat or abdominal fat, they provide no detailed information on body fat distribution or body composition,<sup>36</sup> which may function differently in leading to obesity or its related disorders.<sup>37</sup> For example, subjects with excess visceral fat are reported to be at higher risk for diabetes, hypertension and insulin resistance than those with an excess in subcutaneous fat.<sup>34</sup> It is therefore necessary to conduct more precise measurements using imaging methods, such as magnetic resonance imaging or computed tomographic scanning, to distinguish visceral and subcutaneous adipose tissues<sup>38</sup> in future studies. Fifth, our sample included twins with oversampling of either major depression or PTSD, which may influence the risk of overweight or obesity.<sup>39,40</sup> However, the observed association between aberrant DNA methylation and obese measures is unlikely to be confounded by these two psychiatric conditions because the observed associations persisted after further adjustments for depressive symptoms or PTSD. Sixth, though we were able to control many of the potential confounders, we cannot entirely exclude the possibility of residual confounding by unknown or unmeasured risk factors. Finally, our twin sample was derived from a middle-aged sample of male military veterans; therefore, the generalizability to females and other younger or older populations is not known. Lastly, because our study was cross-sectional, we are unable to determine whether the observed epigenomic signatures are epiphenomena or part of the causal pathways leading to an increased prevalence of obesity.

Nonetheless, this is the first study to demonstrate the association of aberrant DNA methylation in the promoter region of the serotonin transporter gene with obesity in a well-matched MZ twin sample. Previous studies have shown that interindividual epigenetic processes are under genetic control.<sup>41,42</sup> Therefore, the study of the causal impact of epigenotype on disease outcome requires careful matching on or control for genetic background and other potential confounding variables. MZ twin pairs provide a natural experiment to investigate the effects of epigenetics on disease outcome, because MZ twins match exactly on genetic background, age and sex, thus eliminating the effects of these important confounders.<sup>43,44</sup> In addition, identical twins in general share raising environment, providing further control for confounding by early life experience, which has a long-lasting impact on the epigenetic plasticity of human genome.<sup>45</sup>

In summary, this study provides the first evidence that promoter hypermethylation of the serotonin transporter gene is significantly associated with an increased prevalence of obesity. Because epigenetic modifications are possibly reversible, our results, if confirmed, would suggest the potential for lifestyle or therapeutic interventions for obesity, overweight and a wide range of related disorders.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### ACKNOWLEDGEMENTS

This study was supported by Grant 0730100N from the American Heart Association, Grants R21HL092363, K01AG034259, K24HL077506, R01HL68630 and R01AG026255 from the National Institutes of Health. The United States Department of Veterans Affairs has provided financial support for the development and maintenance of the Vietnam Era Twin (VET) Registry. Numerous organizations have provided invaluable assistance in the conduct of this study, including: Department of Defense; National Personnel Records Center, National Archives and Records Administration; the Internal Revenue Service; National Institutes of Health; National Opinion Research Center; National Research Council, National Academy of Sciences and the Institute for Survey Research, Temple University. Most importantly, we gratefully acknowledge the continued cooperation and participation of the members of the VET Registry and their families. Without their contribution this research would not have been possible.

## AUTHOR CONTRIBUTIONS

JZ designed the study, analysed the data and wrote the manuscript. JG contributed to discussion and reviewed/edited the manuscript. VV designed the study, contributed to discussion and reviewed/edited the manuscript.

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