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Original article

Adiposity

Adiposity is associated with DNA methylation profile in adipose tissue

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Abstract

Background: Adiposity is a risk factor for type 2 diabetes and cardiovascular disease, suggesting an important role for adipose tissue in the development of these conditions. The epigenetic underpinnings of adiposity are not well understood, and studies of DNA methylation in relation to adiposity have rarely focused on target adipose tissue. Objectives were to evaluate whether genome-wide DNA methylation profiles in subcutaneous adipose tissue and peripheral blood leukocytes are associated with measures of adiposity, including central fat mass, body fat distribution and body mass index.
Methods: Participants were 106 men and women (mean age 47 years) from the New England Family Study. DNA methylation was evaluated using the Infinium HumanMethylation450K BeadChip. Adiposity phenotypes included dual-energy X-ray absorptiometry-assessed android fat mass, android:gynoid fat ratio and trunk:limb fat ratio, as well as body mass index.
Results: Adipose tissue genome-wide DNA methylation profiles were associated with all four adiposity phenotypes, after adjusting for race, sex and current smoking (omnibus p-values <0.001). After further adjustment for adipose cell-mixture effects, associations with android fat mass, android:gynoid fat ratio, and trunk:limb fat ratio remained. In gene-specific analyses, adiposity phenotypes were associated with adipose tissue DNA methylation in several genes that are biologically relevant to the development of adiposity, such as *AOC3*, *LIPE*, *SOD3*, *AQP7* and *CETP*. Blood DNA methylation profiles were not associated with adiposity, before or after adjustment for blood leukocyte cell mixture effects.
Conclusion: Findings show that DNA methylation patterns in adipose tissue are associated with adiposity.

Key words: Adiposity, adipose tissue, DNA methylation, epigenetics

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Key Messages

- In middle-aged men and women, adipose tissue genome-wide DNA methylation profiles were associated with several measures of adiposity, including centrally-located fat, body fat distribution, and body mass index. Peripheral blood leukocyte DNA methylation was not related to any adiposity phenotype.
- All analyses were adjusted for cell mixture effects, to reduce the potential for confounding by cell mixture in the association between DNA methylation and adiposity. After adipose tissue cell mixture adjustment, adipose tissue DNA methylation remained associated with centrally-located fat and fat distribution, but not with body mass index.
- A stringent gene locus selection criteria was applied to identify genes of interest in the association between adipose tissue DNA methylation and adiposity. Several of the genes selected have strong biological plausibility with respect to adiposity and adipogenesis, and have previously been implicated in human and animal studies of adiposity.

Introduction

Obesity and excess regional fat, often referred to as adiposity,[1] are major risk factors for numerous chronic diseases including type 2 diabetes and cardiovascular disease.[2] Current trends suggest that the total healthcare costs attributable to obesity could reach £610 billion by 2030.[3] The aetiology of adiposity is multifactorial, known to have both genetic[4,5] and environmental determinants.[6,7] Focus has recently turned to improving understanding of underlying molecular mechanisms that may contribute to adiposity, and it is increasingly recognized that adiposity and related cardiometabolic risk may arise as a result of dysregulated cellular programming and alterations of regulatory pathways via epigenetic mechanisms.[8,9] Several prior studies investigated epigenetic contributions to adiposity, as assessed by DNA methylation in humans;[10–20] however, almost all the studies evaluated methylation patterns only in blood.[10–17,19] Given that DNA methylation is a tissue-specific phenomenon, there is considerable interest in investigating directly affected tissues, such as (in this case) adipose tissue. Although epidemiological studies on adipose tissue DNA methylation are starting to increase in number,[18,21,22] little is currently known about associations of adipose tissue DNA methylation with adiposity. Furthermore, even less is known about possible associations of DNA methylation with direct measures of regional fat distribution, particularly centrally-located android fat that has important risks for dyslipidaemia, type 2 diabetes and coronary heart disease.[23,24] Consequently, study objectives were to determine whether epigenome-wide DNA methylation profiles in blood and adipose tissue are cross-sectionally associated with adiposity, including android fat mass, android:gynoid fat ratio, trunk:limb fat ratio and body mass index (BMI).

Research design and methods

Study population

Study participants were from the New England Family Study (NEFS) which comprised 17 921 offspring of pregnant women in the Collaborative Perinatal Project (CPP)[25] from Providence, Rhode Island and Boston, Massachusetts sites (USA),who were recruited between 1959 and 1974. The current NEFS sub-study, termed the Longitudinal Effects on Aging Perinatal (LEAP) Project, includes Providence-born participants selected with oversampling for racial/ethnic minorities and small and large for gestational age. Four hundred participants were enrolled and assessed during 2010–11. Of these, 316 had adequate adipose tissue biopsy performed, 68 refused and 16 had inadequate biopsy specimens. Blood and adipose tissue DNA methylation analyses were performed on a representative sample of 108 of these 316 participants, with adequate DNA methylation data available for 106. Characteristics of participants with DNA methylation data (n = 106) did not differ significantly from those in the overall study sample (n = 400), except that there was a higher percentage of women in the DNA methylation sample (64%) in comparison with the overall study sample (57%).

Measures of adiposity and covariates

Dual-energy X-ray absorptiometry (DXA) scans were performed using the Lunar Prodigy Advance scanner (GE Healthcare, Madison, WI) and provided measures of fat in various body compartments. Adiposity phenotypes of interest included android fat mass (a measure of centrally located fat), android:gynoid region fat mass ratio and trunk:limb region fat mass ratio (as measures of upper:lower

body fat distribution). Weight and height measures were obtained using a calibrated stadiometer by trained personnel, and converted into BMI (kg/m^2). Quality control tests to monitor reproducibility and stability of DXA assessments were performed weekly using models that simulate different levels of body fat. Covariates of interest included race, sex and current smoking. All but 4 participants were White ($n = 72$) or African American ($n = 30$); consequently race was collapsed into 'White' vs 'non-White'.

Tissue sample collection, methylation profiling and data processing

Subcutaneous adipose tissue samples were collected from the upper outer quadrant of the buttock using a 16-gauge needle and disposable syringe. DNA was extracted from adipose tissue samples using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) and the Zymo Genomic DNA Clean & Concentrator Kit, according to the manufacturers' protocol. Whole blood samples were centrifuged to obtain buffy coat, and peripheral blood leukocyte DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), according to the manufacturers' protocol.

DNA was sodium bisulfite-converted using the EZ-96 DNA Methylation-Direct and EZ DNA Methylation-Direct kits (Zymo Research, Orange, CA; as per manufacturer's instructions). Blood and adipose tissue samples were randomly distributed across 18 beadchips on three plates, and analysed using the Infinium Human Methylation450 BeadChip array (Illumina, San Diego, CA) at the Genomics Core Facility at the UCSF Institute for Human Genetics (San Francisco, CA), according to the Illumina protocols for the Infinium platform. Beadchips were scanned with the Illumina iScan system.

Heatmaps of the control probe data and the total signal, as well as the distribution of detection *p*-values were examined to assess data quality. Samples with a detection *p*-value >0.05 for $>1\%$ of probes were excluded from analyses.

The DNA methylation data were out-of-band background corrected and dye bias corrected using the *methylumi* package in R,[26] and further normalized using the Beta-Mixture Quantile Dilation (BMIQ) approach[27] in order to obtain similar ranges for type I vs.type II probes on the Infinium array. For each of the CpG sites and for 834 additional controls, average beta was calculated as $M/(M+U+\varepsilon)$, where M and U refer to the signal from the probe corresponding to the methylated and unmethylated target CpG, respectively, and $\varepsilon = 100$ in order to protect against division-by-zero.

Prior to analyses, 3156 non-CpG probes were excluded, followed by a further exclusion of 11 551 CpG sites located on sex chromosomes, 88 803 CpGs with SNPs in probe[28] (at the flanking G, within 10 nt of the target CpG, and 11-50 nt away from the target CpG), 20 882 probes that are considered cross-reactive[29] and a further 50 probes that had missing values for $>10\%$ of participants. In total, 361 135 CpG sites were included in the analyses.

Statistical analyses

All of the 106 participants with adequate DNA methylation data had available BMI data, while DXA scan data were available for 101 participants. Average beta values were logit-transformed to *M*-values prior to analyses. In a manner similar to combat,[30] *M*-values were adjusted for chip (i.e. beadchip position on plate) effects using a linear mixed effects (LME) model; all chip effects were then subtracted from *M*-values on a CpG-by-CpG basis.

Android fat mass, android:gynoid fat ratio and trunk:limb fat ratio were analysed as continuous variables, with android fat mass and trunk:limb fat ratio log-transformed in order to satisfy assumptions of normality. BMI was analysed as a continuous variable, with a quadratic term in the model to account for non-linearity.

Epigenome-wide association studies

Epigenome-wide association studies (EWAS) analyses involved CpG-by-CpG analysis of *M*-values in combination with omnibus tests for significance via permutation testing (which effectively accounts for multiple testing by controlling for the family-wise error rate). Individual regressions were fit for each CpG (as the dependent variable) in relation to each adiposity phenotype (as the independent variable) with adjustment for sex, race and current smoking. (Analyses were not adjusted for age, given the very narrow age range (44–50 years) in the study sample). Summary of genome-wide association was constructed over all regression coefficients using the minimum nominal *p*-value as the test statistic. Omnibus tests for association were obtained by comparing the test statistic with its corresponding permutation-derived null distribution, generated by permuting phenotype values with respect to DNA methylation data and confounders; 1000 permutations were applied for each test.

EWAS analyses in blood were performed with and without adjustment for blood leukocyte distribution. The method of Houseman *et al.*[31] was first used to estimate effects of phenotype on distribution of the most prevalent distinct leukocyte lineages. For each CpG, effects of leukocyte composition on chip-adjusted average-beta DNA methylation were then determined by a linear regression and subtracted from the corresponding beta values, which

were subsequently logit-transformed back to 'leukocyte-adjusted *M*-values' for analyses.

We also conducted EWAS analyses adjusted for cell mixture effects in adipose tissue, using the recently developed reference-free method of Houseman *et al.*[32] This method adopts a statistical model with a latent variable representing mean methylation, together with a factor-analytical error model similar to the surrogate variable approaches,[33,34] thus producing estimates of non-cell mixture mediated DNA methylation associations. The method requires an assumed latent variable dimension (i.e. number of cell types), which was estimated using the random matrix theory method of Teschendorff *et al.*[34] and was equal to 23 for all analyses.

Given sexual dimorphism in body composition,[35] sex-specific analyses were also performed.

Gene-specific analyses

We conducted gene-specific analyses in order to identify regions of interest with respect to associations with adiposity. For each adiposity phenotype, we conducted CpG-specific analyses (with methylation on the *M*-value scale) and extracted nominal *p*-values corresponding to each CpG-specific regression coefficient. We then summarized this information at the gene region level (i.e. gene regions specified by the manufacturer): for each gene region of a gene, only the CpG with a nominal *p*-value representing the median *p*-value in that gene region was selected for further inquiry. A gene was then selected as a 'top gene of interest' only if it had at least one gene region with a median CpG-specific nominal *p*-value <0.0001, across all adiposity phenotypes analysed. This criterion was implemented for two reasons: (i) selection based on median CpG-specific *p*-value per gene region will mitigate the effects of spurious CpGs that register as an extreme (i.e. outliers); and (ii) an association observed across all adiposity phenotypes utilizes strengths of triangulation, thereby increasing likelihood that findings are reflective of a biologically significant event. It should be noted that all CpGs selected with this approach (i.e. nominal *p*-value of <0.0001) had a false discovery rate (FDR)-corrected *p*-value <0.05 in all analyses.

We separately investigated whether adipose tissue DNA methylation in candidate loci in the *HIF3A* gene were related to BMI in our study sample, in light of recent findings on such an association.[20] We extracted CpG-specific regression results for associations of DNA methylation with BMI, reporting regression coefficients and nominal *p*-values.

All statistical analyses were performed using the R statistical package (v. 3.1.0).

Results

The 106 participants in the study sample had a mean age of 47 (range 44–50) years; 64% were women, 68% White and 34% current smokers. The proportion of women decreased at higher levels of android:gynoid fat ratio, trunk:limb fat ratio, and BMI (Table 1). Mean BMI was positively associated with android fat mass and android:gynoid fat ratio. Adiposity phenotypes were not related to race, years of education or smoking status (Table 1).

EWAS analyses

Adipose tissue DNA methylation profiles were associated with all four adiposity phenotypes in EWAS analyses (omnibus *p*-value <0.001 in all cases) after adjustment for race, sex and current smoking (Table 2). After further adjustment for adipose cell type mixture using the reference-free approach, associations remained with android fat mass, android:gynoid fat ratio and trunk:limb fat ratio but not with BMI (Table 2). Quantile-quantile plots for expected vs observed distribution of *p*-values and estimated genomic inflation factor (i.e. lambda) are available in Supplementary Figure 1a–c (available as Supplementary data at *IJE* online). In sensitivity analyses, android fat mass, android:gynoid fat ratio, and trunk:limb fat ratio remained associated with DNA methylation even after further adjustment for BMI (Supplementary Table 1, available at *IJE* online).

In EWAS analyses in peripheral blood leukocytes, DNA methylation profiles were not related to any adiposity phenotype, whether adjusted or unadjusted for blood leukocyte distribution (Table 3).

We did not observe differences in associations by sex in any of the analyses, therefore only sex-pooled analyses are presented and discussed.

Gene-specific analyses

For associations with android:gynoid fat ratio, android fat mass and trunk:limb fat ratio, we selected genes that contained at least one region with a CpG-specific median *p*-value <0.0001 across all of these adiposity phenotypes; 101 genes met this criterion. For a selection of these genes, Table 4 summarizes associations between adipose tissue DNA methylation and each adiposity phenotype, listing the effect size estimate and nominal *p*-value of the median CpG in each gene region (see Supplementary Table 2 for the full list, available as Supplementary data at *IJE* online).

Finally, we tested whether methylation of adipose tissue at the candidate gene *HIF3A* was associated with BMI, as previously reported.[20] In our analyses we observed the

Table 1. Characteristics of participants, the LEAP Project

	According to tertiles of android fat mass[a]				According to tertiles of android:gynoid fat ratio[a]			
	Tertile 1 (n = 33)	Tertile 2 (n = 34)	Tertile 3 (n = 34)	*p* for trend	Tertile 1 (n = 33)	Tertile 2 (n = 34)	Tertile 3 (n = 34)	*p* for trend
Women, no. (%)	26 (79)	19 (56)	20 (59)	0.09	31 (94)	28 (82)	6 (18)	<.001
White, no. (%)	22 (67)	23 (68)	24 (71)	0.73	24 (73)	20 (59)	25 (74)	0.93
Education, mean years (SD)	13.6 (3.1)	13.4 (3.7)	12.2 (2.4)	0.08	12.7 (2.4)	14 (3.5)	12.5 (3.3)	0.84
Current smoker, no. (%)	12 (36)	10 (29)	14 (41)	0.68	13 (39)	12 (35)	11 (32)	0.55
Ever smoker, no. (%)	18 (54)	18 (53)	24 (71)	0.18	20 (61)	19 (56)	21 (62)	0.91
BMI, mean kg/m^2 (SD)	24.3 (2.8)	31.2 (4.9)	37.4 (5.1)	<.001	28.6 (6.6)	32.1 (8.2)	32.4 (5.1)	0.03

	According to tertiles of trunk:limb fat ratio[a]				According to categorized BMI (kg/m^2)			
	Tertile 1 (n = 33)	Tertile 2 (n = 34)	Tertile 3 (n = 34)	*p* for trend	18.5 - < 25 (n = 24)	25 - < 30 (n = 25)	≥30 (n = 57)	*p* for trend
Women, no. (%)	31 (94)	26 (76)	8 (24)	<.001	20 (83)	15 (60)	33 (58)	0.04
White, no. (%)	20 (61)	24 (71)	25 (74)	0.26	16 (67)	18 (72)	38 (67)	0.92
Education, mean years (SD)	13.3 (2.9)	13.6 (3.2)	12.3 (3.3)	0.20	14.5 (3.3)	13.2 (2.4)	12.7 (3.4)	0.18
Current smoker, no. (%)	10 (30)	14 (41)	12 (35)	0.68	9 (38)	9 (36)	18 (32)	0.58
Ever smoker, no. (%)	16 (49)	21 (62)	23 (68)	0.11	13 (54)	13 (52)	35 (61)	0.47
BMI, mean kg/m^2 (SD)	30.9 (9.1)	29.5 (5.5)	32.9 (5.2)	0.23	22.9 (1.5)	27.6 (1.6)	36.6 (5.3)	<.001

[a]Tertiles for android fat mass, android:gynoid fat ratio, and trunk:limb ratio add up to 101, as only 101 of the 106 participants had DXA scan measures available.

Table 2. EWAS analyses:[a] adipose tissue DNA methylation in relation to adiposity phenotypes, the LEAP Project

	Analyses not adjusted for cell mixture effects	Analyses adjusted for cell mixture effects
	Omnibus *p*-values[b] Based on permutation test	Omnibus *p*-values[b] Based on reference-free approach
Android:gynoid fat ratio	<0.001	0.024
Android fat mass	<0.001	0.003
Trunk:limb fat ratio	<0.001	0.011
Body mass index	<0.001	0.195

[a]Analyses on android fat mass, and trunk:limb and android:gynoid fat ratios include 101 participants, whereas analyses on BMI include 106. All analyses adjusted for sex, race (White vs non-White) and current smoking.

[b]Analyses not adjusted for cell mixture report omnibus *p*-values based on permutation tests, using the minimum *p*-value test statistic to construct summary of genome-wide associations. Analyses adjusted for cell mixture, using a reference-free method, report omnibus *p*-values based on the bootstrap method described in Houseman *et al*. 2014.[32]

Table 3. EWAS analyses:[a] peripheral blood leukocyte DNA methylation in relation to adiposity phenotypes, the LEAP Project

	Analyses not adjusted for leukocyte distribution	Analyses adjusted for leukocyte distribution
	Omnibus *p*-values[b] Based on permutation test	Omnibus *p*-values[b] Based on permutation test
Android:gynoid fat ratio	0.408	0.635
Android fat mass	0.688	0.802
Trunk:limb fat ratio	0.460	0.058
Body mass index	0.738	0.954

[a]Analyses on android fat mass and trunk:limb and android:gynoid fat ratios include 101 participants, whereas analyses on BMI include 106. All analyses adjusted for sex, race (White vs non-White) and current smoking.

[b]Analyses report omnibus *p*-values based on permutation tests, using the minimum *p*-value test statistic to construct summary of genome-wide associations. Analyses adjusted for cell mixture are based on the method described in Houseman *et al*. 2012,[31] using DNA methylation measures at 100 select DMRs as surrogate markers of leukocyte distribution.

same three CpGs reported by Dick *et al*. to be associated with BMI [nominal *p*-value = 1.3×10^{-6}, 4.3×10^{-6} and $5.5 \times 10^{-5}$for cg27146050, cg22891070 and cg16672562, respectively (see Table 5)]. It should be noted, however, that these associations did not meet the Bonferroni correction threshold ($0.05/361\,135 = 1.4 \times 10^{-7}$) for our analyses. Blood DNA methylation at these CpG sites was not related to BMI in our analyses.

Table 4. Adipose tissue gene-specific DNA methylation in relation to adiposity, the LEAP Project (select number of genes displayed; full list of all 101 genes available in Supplementary Table 2, available at *IJE* online)

	Median, CpG-specific effect size estimate (*p*-value)[a] per gene region[b]		
	Android:gynoid fat ratio	Android fat	Trunk:limb fat ratio
ADAMTS4			
5'UTR	0.107 (5.6×10^{-6})	0.036 (1.3×10^{-6})	0.1 (2.0×10^{-6})
TSS1500	0.092 (2.1×10^{-4})	0.027 (5.2×10^{-4})	0.092 (3.3×10^{-5})
TSS200	0.143 (5.9×10^{-4})	0.058 (1.9×10^{-5})	0.136 (2.6×10^{-4})
1stExon	0.107 (5.6×10^{-6})	0.036 (1.3×10^{-6})	0.1 (2.0×10^{-6})
3'UTR	0.051 (0.04)	0.02 (0.01)	0.04 (0.07)
AGAP2			
TSS1500	0.024 (9.3×10^{-3})	0.006 (3.3×10^{-3})	0.013 (9.2×10^{-3})
TSS200	0.035 (8.6×10^{-3})	0.019 (1.6×10^{-3})	0.032 (0.03)
1stExon	0.117 (2.0×10^{-4})	0.049 (7.9×10^{-7})	0.118 (2.4×10^{-5})
Body	0.137 (2.3×10^{-4})	0.056 (2.8×10^{-6})	0.126 (5.1×10^{-5})
3'UTR	0.201 (1.7×10^{-5})	0.075 (2.9×10^{-7})	0.163 (1.2×10^{-4})
ANGPT2			
5'UTR	0.124 (4.0×10^{-4})	0.044 (7.4×10^{-5})	0.128 (3.8×10^{-5})
TSS1500	0.088 (1.9×10^{-3})	0.02 (0.03)	0.063 (0.01)
TSS200	0.122 (3.27×10^{-4})	0.046 (1.5×10^{-5})	0.123 (4.3×10^{-5})
1stExon	0.142 (2.1×10^{-4})	0.051 (3.7×10^{-5})	0.143 (2.0×10^{-5})
Body	0.106 (1.3×10^{-3})	0.031 (2.1×10^{-3})	0.091 (2.2×10^{-3})
ANGPT4			
5'UTR	0.145 (2.1×10^{-6})	0.046 (1.5×10^{-6})	0.157 (3.6×10^{-9})
TSS1500	0.132 (1.1×10^{-4})	0.049 (4.4×10^{-6})	0.151 (4.4×10^{-7})
TSS200	0.04 (2.7×10^{-4})	0.01 (5.5×10^{-3})	0.048 (1.7×10^{-7})
1stExon	0.145 (2.1×10^{-6})	0.046 (1.5×10^{-6})	0.157 (3.6×10^{-9})
Body	0.024 (0.10)	0.007 (0.07)	0.021 (0.09)
AOC3			
5'UTR	0.15 (1.4×10^{-4})	0.056 (4.6×10^{-6})	0.147 (2.8×10^{-5})
TSS1500	0.052 (0.04)	0.023 (1.4×10^{-3})	0.047 (0.02)
TSS200	0.137 (8.3×10^{-5})	0.053 (8.5×10^{-7})	0.137 (9.3×10^{-6})
1stExon	0.15 (7.3×10^{-5})	0.056 (1.8×10^{-6})	0.147 (1.1×10^{-5})
Body	0.187 (1.1×10^{-5})	0.066 (8.4×10^{-7})	0.186 (7.9×10^{-7})
3'UTR	0.076 (2.5×10^{-3})	0.035 (4.8×10^{-6})	0.066 (3.8×10^{-3})
AQP7			
5'UTR	0.108 (3.5×10^{-5})	0.037 (5.5×10^{-6})	0.117 (3.1×10^{-7})
1stExon	0.108 (3.4×10^{-5})	0.037 (5.4×10^{-6})	0.117 (3.1×10^{-7})
Body	0.023 (0.19)	0.006 (0.31)	0.02 (0.19)
CETP			
5'UTR	0.124 (7.6×10^{-5})	0.039 (4.4×10^{-5})	0.121 (5.4×10^{-6})
TSS1500	0.149 (6.8×10^{-5})	0.045 (7.0×10^{-4})	0.148 (4.5×10^{-6})
TSS200	0.098 (2.5×10^{-4})	0.028 (1.2×10^{-3})	0.099 (3.4×10^{-5})
1stExon	0.124 (7.6×10^{-5})	0.039 (4.4×10^{-5})	0.121 (5.4×10^{-6})
Body	0.004 (0.44)	−0.002 (0.01)	0.004 (0.14)
3'UTR	0.128 (3.2×10^{-7})	0.032 (1.0×10^{-4})	0.115 (2.9×10^{-7})
DOCK9			
5'UTR	0.215 (1.9×10^{-6})	0.073 (1.9×10^{-7})	0.196 (1.2×10^{-6})
TSS1500	0.052 (0.01)	0.014 (0.20)	0.053 (1.7×10^{-3})
TSS200	0.225 (8.7×10^{-6})	0.077 (8.1×10^{-7})	0.212 (2.8×10^{-6})
1stExon	0.107 (0.46)	0.037 (0.46)	0.098 (0.40)
Body	0.027 (0.07)	0.009 (0.04)	0.034 (0.04)
3'UTR	0.006 (0.51)	0.002 (0.25)	-2×10^{-5} (0.30)
HOXA3			
5'UTR	0.051 (0.13)	0.017 (0.08)	0.033 (0.25)
TSS1500	0.056 (0.13)	0.019 (0.07)	0.034 (0.28)

(Continued)

Table 4. Continued

	Median, CpG-specific effect size estimate (*p*-value)[a] per gene region[b]		
	Android:gynoid fat ratio	Android fat	Trunk:limb fat ratio
TSS200	0.251 (4.8×10^{-7})	0.089 (3.6×10^{-9})	0.213 (3.3×10^{-6})
1stExon	0.113 (4.3×10^{-5})	0.044 (8.6×10^{-8})	0.115 (2.3×10^{-6})
Body	0.006 (0.04)	0.001 (0.23)	0.006 (0.03)
3'UTR	0.112 (2.6×10^{-3})	0.028 (9.0×10^{-3})	0.101 (9.6×10^{-4})
LIPE			
5'UTR	0.21 (4.2×10^{-6})	0.059 (5.6×10^{-5})	0.208 (2.6×10^{-7})
TSS1500	−0.001 (0.13)	0.001 (0.30)	−0.001 (0.46)
TSS200	0.289 (4.9×10^{-7})	0.09 (8.4×10^{-7})	0.29 (1.1×10^{-8})
1stExon	0.21 (4.2×10^{-6})	0.059 (5.6×10^{-5})	0.208 (2.6×10^{-7})
Body	0.082 (1.7×10^{-4})	0.033 (4.9×10^{-5})	0.066 (1.6×10^{-4})
3'UTR	−0.005 (0.32)	−0.003 (0.09)	−0.007 (0.13)
SOD3			
5'UTR	0.079 (0.39)	0.033 (0.06)	0.075 (0.10)
TSS1500	0.054 (6.6×10^{-3})	0.025 (4.1×10^{-4})	0.068 (1.8×10^{-4})
TSS200	0.183 (6.7×10^{-6})	0.064 (7.0×10^{-7})	0.185 (3.7×10^{-7})
1stExon	0.155 (4.0×10^{-6})	0.059 (1.1×10^{-8})	0.152 (3.7×10^{-7})
Body	0.027 (0.32)	0.002 (0.52)	0.022 (0.36)
3'UTR	0.018 (1.4×10^{-4})	0.002 (0.16)	0.017 (4.3×10^{-5})
TIMP4			
5'UTR	0.113 (1.6×10^{-5})	0.026 (1.8×10^{-4})	0.102 (1.5×10^{-5})
TSS1500	0.191 (2.7×10^{-7})	0.064 (3.7×10^{-8})	0.184 (2.3×10^{-8})
TSS200	0.137 (1.3×10^{-6})	0.041 (4.8×10^{-6})	0.123 (1.2×10^{-6})
1stExon	0.113 (1.6×10^{-5})	0.026 (2.5×10^{-3})	0.102 (3.0×10^{-5})
Body	0.2 (0.01)	0.043 (0.27)	0.239 (1.6×10^{-4})
3'UTR	0.018 (0.12)	0.006 (0.11)	0.026 (0.01)

UTR, untranslated region; TSS, transcription start site.

[a]For each of the adiposity phenotypes (columns), results report the effect size estimate and nominal *p*-value for the CpG with the median nominal *p*-value in each gene region of the gene. Although analyses for the process of gene selection were based on models with DNA methylation on the M-value scale, once the top genes of interest were selected, results for the top genes were redone with methylation on the beta scale, in order to provide interpretable effect estimates in this table. Therefore, results displayed here are based on models with the adiposity phenotype of interest as exposure, DNA methylation (on the beta scale) as the outcome and adjusted for sex, race and current smoking.

[b]For some genes, not all gene regions are displayed, as no CpGs (after applying our CpG exclusion criteria) were mapped to that gene region.

Discussion

Overall, in this study of middle-aged men and women, adipose tissue DNA methylation profiles were associated with BMI, centrally-located android fat mass and fat distribution measures of android:gynoid and trunk:limb fat ratios, after adjustment for sex, race and current smoking. Further adjustment for adipose cell mixture attenuated associations with BMI. There was no evidence of associations between peripheral blood leukocyte DNA methylation and adiposity. This suggests that there is important tissue specificity in the origin of adiposity-related DNA methylation.

We implemented a stringent gene locus selection criterion to identify genes where DNA methylation was associated with adiposity. Several of these genes have strong biological plausibility with respect to adipogenesis and adiposity, including *AOC3*,[36,37] *SOD3*,[38–41] *DOCK9*,[42,43] *AQP7*,[44–49] *ANGPT4*,[50] *ANGPT2*,[50–52] *TIMP4*[53–57] and *ADAMST4*.[50] For example, *AOC3* encodes a major protein that resides on the adipocyte plasma membrane,[37] and serum levels of the protein predicted 10-year cardiovascular mortality in type 2 diabetic subjects.[36] *SOD3,* which encodes an antioxidant enzyme,[39,40,58] has been related to BMI in type 2 diabetes patients[38] and is responsive to high-fat feeding in white adipose tissue of male rats.[59] *AQP7*encodes a glycerol transporter expressed in adipocytes, and its regulation is thought to be crucial for glycaemia control.[46,60] Several lines of evidence from both animal[44,45] and human[48,49] studies suggest that down-regulation of *AQP7* is associated with development of obesity. Specifically, *AQP7* gene expression was shown to be down-regulated in subcutaneous adipose tissue of obese vs lean individuals.[48] *TIMP4, ANGPT2, ANGPT4* and *ADAMTS4* encode proteins involved in processes that

Table 5. Adipose tissue CpG DNA methylation in the *HIF3A* gene in relation to body mass index, the LEAP Project

CpG locus	Beta coefficient	Nominal *p*-value
cg07022477	7.5×10^{-4}	0.29
cg19045239	-1.45×10^{-3}	0.07
cg15229275	-1.47×10^{-3}	0.24
cg19310908	2.8×10^{-4}	0.38
cg14117138	3.3×10^{-4}	0.27
cg10594090	-2.3×10^{-4}	0.38
cg14088357	1.0×10^{-3}	0.38
cg02879662	1.7×10^{-4}	0.63
cg09789590	6.8×10^{-4}	0.31
cg05286653	1.8×10^{-3}	0.19
cg27146050[a]	2.7×10^{-3}	1.3×10^{-6}
cg22891070[a]	5.8×10^{-3}	4.3×10^{-6}
cg16672562[a]	3.7×10^{-3}	5.5×10^{-5}
cg12068280	3.3×10^{-3}	3.8×10^{-5}
cg21617218	-9.9×10^{-5}	0.07
cg11253785	-2.6×10^{-5}	0.63
cg01552731	8.4×10^{-4}	0.23
cg25196389	1.1×10^{-3}	0.03
cg23548163	2.8×10^{-3}	0.01
cg26749414	1.1×10^{-3}	0.10
cg20667364	2.5×10^{-3}	0.02
cg07684068	1.1×10^{-3}	0.01
cg20969614	1.0×10^{-3}	0.58
cg25460031	3.8×10^{-4}	0.47
cg14153927	5.7×10^{-4}	0.25

CpGs are listed in order according to their position on the genome, based on annotation information provided by Illumina.

Analyses are based on models with methylation on the beta scale, adjusting for sex, race and current smoking.

[a]CpG loci reported to be associated with BMI in the study by Dick *et al.* 2014.[20]

modulate adipose tissue structure during development of adiposity, including adipogenesis, angiogenesis and extracellular matrix remodelling.[50] In particular, altered levels of ANGPT2 protein have been observed in subcutaneous adipose tissue[52] and serum[51] of obese and overweight vs lean individuals. *TIMP4* encodes a protein that interacts with matrix metalloproteinases, which are active in human adipocytes and have been associated with obesity in rodents and humans.[53–57]

In addition, genes related to fatty acid and lipid metabolism were also identified in our list of top genes, including *LIPE* and *CETP*. *LIPE,* also known commonly as hormone-sensitive lipase (HSL), encodes a protein expressed in adipose tissue and the heart where it primarily hydrolyzes stored triglycerides to free fatty acids. LIPE is a key enzyme in adipocyte lipolysis, and alterations in lipolysis have been frequently associated with obesity.[61] *LIPE* expression and function in subcutaneous fat cells were observed to be associated with obesity among a sample of men and women.[62] *CETP* encodes a protein that plays a key role in lipid metabolism and was one of the top differentially expressed genes in adipose tissue in a study of diet-induced weight loss among obese subjects.[63] *CETP* has been related to atherosclerosis, type 2 diabetes and obesity.[64–67]

A prior investigation reported blood DNA methylation at three CpG sites, located in the *HIF3A* gene, to be associated with BMI among 479 individuals, as well as in a primary and secondary replication cohort. DNA methylation at these three CpG sites was also shown to be positively associated with BMI in subcutaneous adipose tissue of 635 White females (reported *p*-values of 9.27×10^{-7}, 1.72×10^{-5} and 5.01×10^{-6} for cg27146050, cg22891070 and cg16672562, respectively).[20] Interestingly, we found that associations of subcutaneous adipose tissue DNA methylation at these same three CpG loci were positively associated with BMI in our study sample. *HIF3A* encodes a protein that is one component of the heterodimeric hypoxia-inducible factor (HIF) transcriptional complex, which regulates many adaptive responses to hypoxia.[68,69] The specific role of *HIF3A* in adiposity is not well known, but adipocyte-specific targeted disruption of other genes (*HIF1A* and *ARNT*) in the HIF heterodimer was associated with reduced fat formation and insulin resistance in transgenic mice fed a high-fat diet, in comparison with wild-type control mice who were also fed a high-fat diet.[70]

Several recent publications have highlighted the importance of adjusting for cell type composition in generating DNA methylation profiles that reflect phenotypic differences,[32,71,72] given that cell type composition is related both to DNA methylation signatures[73] and disease states.[71,74,75] Similar to blood, cell mixture may also be important to consider in adipose tissue analyses, since this tissue is also heterogeneous, and the cellular composition of adipose tissue can shift as a result of adiposity and related inflammation.[76,77] Whereas adjustment for cell mixture in blood DNA methylation analyses is facilitated by the availability of reference datasets on cell types in blood,[31] such reference datasets are not available for other tissue types such as adipose tissue. Recently developed statistical methods, however, allow for cell mixture adjustment without the need to rely on such reference datasets on underlying cell types.[32,72] We thus implemented the reference-free method of Houseman *et al.*[32] to adjust for cell mixture effects in our adipose tissue analyses, and found that all adiposity phenotypes, except for BMI, remained associated with genome-wide DNA methylation. Thus, associations of DNA methylation with BMI may potentially be due to cell mixture effects, whereas associations observed with direct, DXA-assessed measures of fat suggest that regulatory mechanisms involving DNA methylation in genes or pathways are related to development of adiposity.

DNA methylation profiles in peripheral blood leukocytes were not related to adiposity in the current study. This result is consistent with some prior studies[13,15,19] but in contrast with others.[10–12,14,16,20] Heterogeneity in study populations, methylation profiling methods, analytical approaches and assessments of cell mixture across studies may be causes of differences in findings.[75]

Strengths of this study include the assessment and analysis of genome-wide DNA methylation performed in both peripheral blood leukocytes and adipose tissue. Furthermore, we were able to assess DNA methylation in relation to direct measures of central fat and body fat distribution, in addition to BMI. Finally, analyses adjusted for cell mixture effects in both blood and adipose tissue.

With regard to limitations, DNA methylation was assessed at a single time point in adulthood, and concurrently with the adiposity outcomes of interest. Consequently, directionality or causality cannot be inferred from the associations observed. In addition, low power due to study sample size is possible, and may be one reason for lack of associations in blood. Furthermore, future validation and replication attempts regarding findings in this study are important, to establish whether observed associations are accurate and generalizable.

In conclusion, this study found adipose tissue DNA methylation to be related to several different measures of adiposity. This provides strong support for a novel epigenetic mechanism, potentially affected by both environmental and genetic factors in development, to contribute to adiposity.

Supplementary Data

Supplementary data are available at *IJE* online.

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