



ORIGINAL ARTICLE

Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci

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Abstract

Obesity is an important component of the pathophysiology of chronic diseases. Identifying epigenetic modifications associated with elevated adiposity, including DNA methylation variation, may point to genomic pathways that are dysregulated in numerous conditions. The Illumina 450K Bead Chip array was used to assay DNA methylation in leukocyte DNA obtained from 2097 African American adults in the Atherosclerosis Risk in Communities (ARIC) study. Mixed-effects regression models were used to test the association of methylation beta value with concurrent body mass index (BMI) and waist circumference (WC), and BMI change, adjusting for batch effects and potential confounders. Replication using whole-blood DNA from 2377 White adults in the Framingham Heart Study and CD4+ T cell DNA from 991 Whites in the Genetics of Lipid Lowering Drugs and Diet Network Study was followed by testing using adipose tissue DNA from 648 women in the Multiple Tissue Human Expression Resource cohort. Seventy-six BMI-related probes, 164 WC-related probes and 8 BMI change-related probes passed the threshold for significance in ARIC ($P < 1 \times 10^{-7}$; Bonferroni), including probes in the recently reported *HIF3A*, *CPT1A* and *ABCG1* regions. Replication using blood DNA was achieved for 37 BMI probes and 1 additional WC probe. Sixteen of these also replicated in adipose tissue, including 15 novel methylation findings near genes involved in lipid metabolism, immune response/cytokine signaling and other diverse pathways, including *LGALS3BP*, *KDM2B*, *PBX1* and *BBS2*, among others. Adiposity traits are associated with DNA methylation at numerous CpG sites that replicate across studies despite variation in tissue type, ethnicity and analytic approaches.

Introduction

Epigenetics is the study of mitotically heritable modifications in chromatin structure not involving the underlying DNA sequence, and their impact on the transcriptional control of genes and cellular function. Of the different forms of epigenetic modification, DNA methylation is the most extensively studied and involves the addition and removal of methyl (-CH₃) groups at CpG dinucleotides to influence regional DNA transcription (1). Although genome-wide demethylation and re-methylation occur during embryogenesis and established patterns must be set to initiate differentiation and maintain cell type-specific gene expression (2), DNA methylation and other features of the epigenome are also modifiable throughout the life course by environmental and behavioral exposures such as the nutrient content of the maternal diet (3), cigarette smoking (4) and environmental pollutants (5). Because of their role in gene expression, alterations in epigenetic patterns are a mechanism by which these and other environmental factors may increase risk of disease (6,7).

As individuals accrue excess adipose tissue, they experience chronic low-grade inflammation, associated with immunological activation and oxidative stress (8,9), as well as insulin resistance, hypertension and dyslipidemia (10). These features explain, in part, why obesity is among the strongest modifiable risk factors for diabetes, atherosclerosis and some cancers (11–19). In an epigenetic framework, obesity can be seen as an environmental factor that exposes the genome in many tissues to a suite of systemic factors [e.g. elevated circulating C-reactive protein, interleukin (IL) 6], potentially altering DNA methylation or histone protein acetylation patterns. Identifying epigenetic modifications associated with obesity may therefore point to genomic pathways that are dysregulated in numerous conditions.

As of 2015, only a small number of studies had been published showing obesity-related variation in DNA methylation, with most studies generally using either targeted repeat sequence (global methylation) or candidate gene-centric approaches. With some exceptions (20,21), such studies have not yielded results that have been replicated in independent cohorts (reviewed by Drong *et al.*) (7). Recent technological advances have provided platforms for systematically interrogating DNA methylation variation across the genome (22), paving the way for epigenome-wide association studies (EWASs), analogous to genome-wide association studies, to identify regions of the genome-harboring DNA methylation variation associated with disease phenotypes (6).

EWASs of obesity traits have shown that methylation variants are influenced by nearby genetic variants (i.e. are haplotype specific) as in the case of the well-documented obesity gene *FTO* (23–26). To date, only one obesity EWAS has yielded a novel replicated locus (CpG sites in *HIF3A*) (27), and only one obesity EWAS has been conducted in African Americans (25), despite the fact that African-ancestry groups tend to carry higher chronic disease risk factor loads, including greater obesity, compared with European-ancestry populations (28–30).

The goal of the present investigation is to advance our understanding of the methylation signatures associated with obesity traits using leukocyte DNA samples from over 2000 African American adults, many of whom were overweight or obese at the time of DNA collection, with replication in three independent cohorts. Our findings replicate those from recent EWAS of body mass index (BMI), lipid and diabetes-related traits and identify a number of novel associations with BMI, waist circumference (WC) and BMI change. The results are a step toward understanding the pathophysiology of obesity and identifying new molecular targets to avert its negative health consequences.

Results

Description of discovery sample

The ARIC Study is a prospective cohort study of cardiovascular disease risk in White and Black adults from four US communities (31). Subjects were seen at baseline (Visit 1) in 1987–1989, with four follow-up visits (Visits 2–5) thereafter. The study sample for the present investigation ($N = 2097$; 64% female) includes only those with methylation data (all of whom are African American). Average age was 56 years at the time of Visit 2 when DNA methylation data and adiposity measures were both available. Subjects had mean BMI, WC and BMI change of 30.1 kg/m², 101.3 cm and 7.0 kg/m² (6.0), respectively. Most of the subjects were overweight (37%) or obese (44%) and 67% exceeded National Heart, Lung, and Blood Institute (NHLBI)-recommended WC limits (>88 cm for women and >102 cm for men). Prevalent diabetes was present in 26% of the participants. Imputed white blood cell (WBC) count differentials were obtained for all subjects, and the mean proportions of each cell type as well as other study covariates are provided in Table 1. A flowchart (Fig. 1) outlines the results of the subsequent analyses, detailed later.

Table 1. Characteristics of the study sample: N = 2097 African American adults in the ARIC study

	Mean (SD)	Range	N
Age (years)	56.2 (5.7)	47–70	2097
BMI (kg/m ²)	30.1 (6.1)	14.7–62.4	2096
WC (cm)	101.3 (15.1)	61–163	2097
BMI change (kg/m ²)	7.0 (6.0)	–25.9–36.4	2039
Physical activity (scale of 1–5) ^a	2.1 (0.6)	1–4.25	2097
WBC count (1000/mm ³)	5.6 (1.9)	2.0–28.8	2097
	N (proportion)		
Sex			
Male	763 (0.36)		2097
Female	1334 (0.64)		
Field center			
Forsyth County, NC	157 (0.07)		2097
Jackson, MS	1940 (0.93)		
BMI status ^b			
Underweight (BMI < 18.5 kg/m ²)	19 (0.01)		2095
Normal weight (BMI 18.5–24.99 kg/m ²)	369 (0.18)		
Overweight (BMI 25.0–29.99 kg/m ²)	789 (0.37)		
Obese (≥30.0 kg/m ²)	918 (0.44)		
WC status ^b			
Normal	698 (0.33)		2097
Elevated	1399 (0.67)		
Cigarette smoking			
Current smoker	512 (0.24)		2097
Current nonsmoker	1585 (0.76)		
Alcohol use			
Current drinker	717 (0.34)		2097
Current nondrinker	1380 (0.66)		
Education			
<High school	843 (0.40)		2097
High school graduate	581 (0.28)		
>High school	673 (0.32)		
Household income			
<\$16 000	1087 (0.52)		2097
\$16 000–\$24 999	379 (0.18)		
\$25 000–\$34 999	278 (0.13)		
\$35 000–\$49 999	202 (0.10)		
>\$50 000	151 (0.07)		
Differential WBC proportions ^c			
Neutrophils	0.56		2097
Lymphocytes	0.36		
Monocytes	0.05		
Eosinophils	0.03		
Diabetes status ^d			
No	1539 (0.74)		2087
Yes	548 (0.26)		

^aSelf-reported leisure time physical activity using the Baecke Questionnaire at Visit 1.

^bBMI status: underweight (BMI <18.5 kg/m²), normal weight (BMI 18.5–24.99 kg/m²), overweight (BMI 25.0–29.99 kg/m²) and obese (≥30.0 kg/m²). WC status: low risk (WC ≤88 cm for women and ≤102 cm for men) and high risk (WC >88 cm for women and >102 cm for men).

^cDifferential WBC proportions were imputed using the method of Houseman *et al.* (32) using measured differentials from a subset of 179 ARIC subjects as the reference.

^dPrevalent diabetes at the time of the DNA collection was defined as a self-reported physician diagnosis of or treatment for diabetes.

Gene pathway analysis

To test the global hypothesis that there are significant associations of adiposity traits with methylation variation, we conducted a genome-wide gene ontology (GO) pathway analysis for BMI and WC including all CpGs, using the same statistical models as in the subsequent EWAS. The approach accounted for the degree of clustering of probes included in the HM450 in particular genes and gene regions. Results yielded significant [false discovery rate (FDR)-adjusted q -value < 0.05] enrichment of over 100 biological process pathways for BMI and 90 biological process pathways for WC (Supplementary Material, Tables S1 and S2),

involving 11–204 genes per pathway. These pathways represent a diverse range of processes, particularly related to neuronal function (e.g. neuron migration and neuroblast proliferation), immune response/cytokine signaling (e.g. humoral immune response and positive regulation of IL-6 production) and energy and fatty acid metabolism [e.g. tricarboxylic acid (Krebs') cycle and arachidonic acid secretion].

Association study

Manhattan plots showing the $-\log_{10}$ P-values for individual autosomal CpG probe associations for BMI and WC are provided in

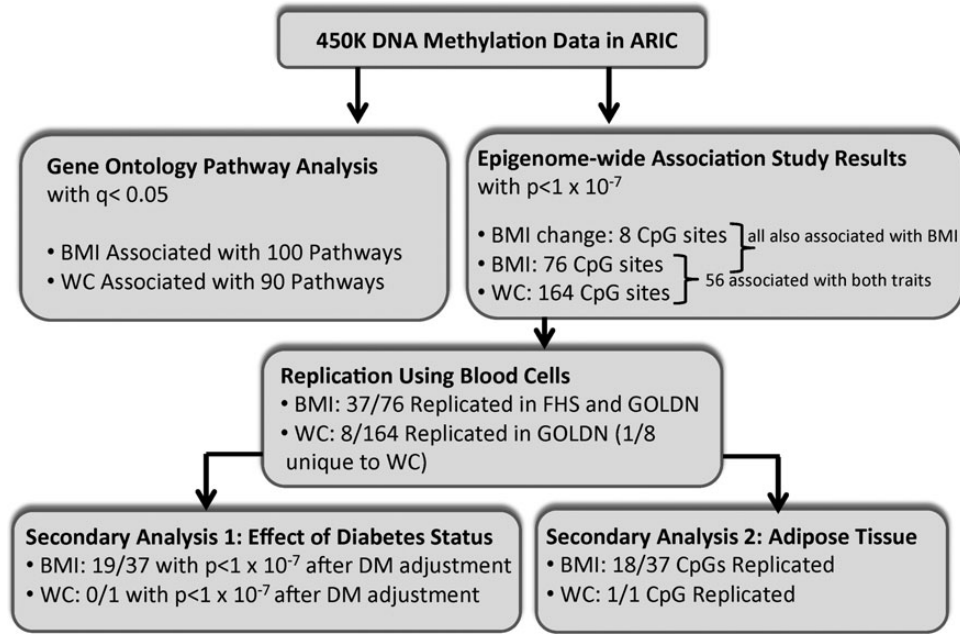


Figure 1. Flowchart of experiments and summary of results.

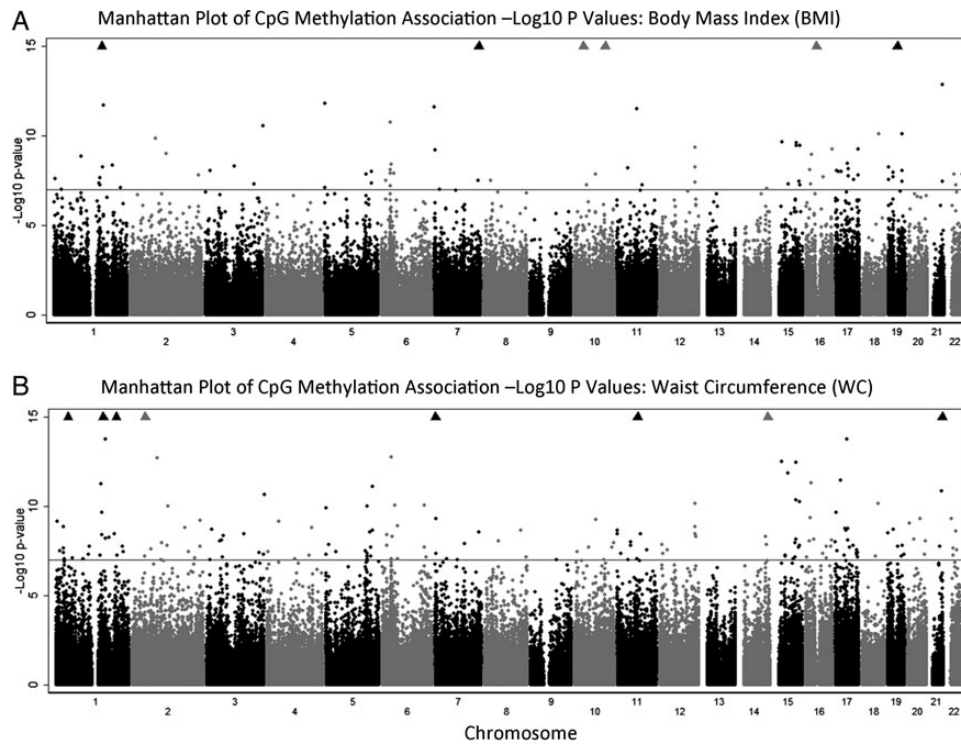


Figure 2. Manhattan plot of CpG methylation association $-\log_{10}$ P-values in 2107 African American adults in the ARIC study. (A) BMI and (B) WC.

Figure 2. A total of 76 probes passed the threshold for genome-wide significance for BMI, and 164 probes passed this threshold for WC. For BMI change, eight probes passed this threshold, all of which were also among the statistically significant BMI and/or WC probes ($P < 1 \times 10^{-7}$) (Supplementary Material, Table S3), including cg15871086 (Chr. 18 intergenic, $P = 8.77E - 10$), cg09554443 (near *CD247*, $P = 2.68E - 09$), cg00574958 [near carnitine palmitoyltransferase-1A (*CPT1A*), $P = 4.30E - 08$] and

cg16672562 [near hypoxia-inducible factor 3 (*HIF3A*), $P = 8.60E - 08$]. *CPT1A* methylation probe associations have been recently reported to be associated with atherogenic lipoprotein subfractions in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) cohort (33). Methylation at cg16672562 near *HIF3A* was recently reported to be associated with BMI in ~2500 European adults in the Cardiogenetics Consortium, MARseille THrombosis Association and Cooperative Health Research in the Region of

Augsburg cohorts (27). BMI change associations are not discussed in further detail due to their complete overlap with BMI and WC results.

Regression coefficients, standard errors, *P*-values and CpG marker information for the significant associations for BMI and WC in the ARIC discovery sample are provided in Supplementary Material, Tables S4 and S5, respectively. For BMI, the top CpG (cg06500161, $P = 1.52E - 13$) explained ~2.6% of variation in BMI and is located in a CpG island shore in the gene body of *ABCG1* [ATP-binding cassette, subfamily G (WHITE), member 1]. This gene is expressed in blood plasma and platelets and is involved in macrophage cholesterol and phospholipids transport, and cellular regulation of lipid homeostasis (34). *ABCG1* promoter hypermethylation is strongly associated with coronary heart disease (CHD) (35), and this particular CpG was also associated with insulin-related traits in the GOLDN cohort (33). A second CpG site near *ABCG1* also was among the top results (cg27243685, $P = 3.61E - 08$). The two CpGs are ~14 kb distant from one another; their mean methylation beta values were significantly different from one another (mean beta value for cg06500161 = 0.62; mean beta value for cg27243685 = 0.85; unpaired *t*-test *P*-value < 0.0001) and were moderately inter-correlated ($r = 0.32$; $P < 0.0001$). Other loci exhibiting two or more significant CpG associations included the smoking-related methylation locus *AHRR* (cg23576855 and cg05575921), *HIF3A* (cg27146050, cg22891070 and cg16672562) with the same three CpG sites previously reported by Dick et al. (27) to be associated with BMI, the innate immune response regulator *NFKBIL1* (cg21053741 and cg21587837), the histone H3 demethylase *KDM2B* (cg15695155, cg26995224 and cg13708645), the natural killer immune response gene *LGALS3BP* (cg04927537 and cg25178683) and *NWD1* of unknown function (cg15845821 and cg19344626). Conditional regression models (which required switching BMI to the dependent variable) were run for loci with >1 CpG association and showed no evidence for multiple independent signals within these loci. After *ABCG1* and *AHRR*, the third-ranked CpG association was near the *CD247* gene, involved in T-cell receptor signaling, immune response and IL-12-induced interferon gamma ($IFN\gamma$) production. As a note, it is likely that the observed CpG associations near *AHRR* were the result of residual confounding; our EWAS was conducted using smoking coded as current/noncurrent, and when we further adjusted the results for former smoking and total pack years of smoking, the associations with BMI were greatly attenuated and no longer statistically significant (data not shown).

There was considerable overlap between results for BMI and WC; 56 of the BMI associations (74%) were also significant for WC and the top CpG was the same for both traits (cg06500161 near *ABCG1*, *P* for WC = 4.41E - 19). This CHD-associated marker explained ~3.6% of variation in WC. The second-ranked probe in the WC analysis was cg00574958 ($P = 5.79E - 17$) near *CPT1A*, also mentioned earlier. Additional high-ranking probes in our WC EWAS included a site near *LY6G6E* (cg13123009, $P = 1.8 \times 10^{-13}$), which belongs to a cluster of leukocyte antigen-6 (*LY6*) genes located in the major histocompatibility complex (MHC) class III region on chromosome 6, and *C7orf50*, a longevity locus (36). It is generally noted that probes passing the threshold for significance for BMI and WC in ARIC explained 0.2–3.6% trait variance, included CpG sites across the spectrum of average methylation values (minimum 0.04 to maximum 0.95 mean methylation) and had intra-class correlation (ICC) coefficient values for replicates of >0.35 (Supplementary Material, Tables S4 and S5). Most CpG markers had ICC > 0.60, suggesting higher probe reliability/quality as measured by ICC increases the probability of detecting association.

Replication of association results

We took forward the 76 BMI-associated probes for replication in the Framingham Heart Study (FHS) (methylation data for which subjects were generated in one of two laboratories and considered separately here) and GOLDN. Details on the replication cohorts are found in the Supplementary Text. Direction and *P*-value for each BMI probe are provided by cohort, and meta-analysis *P*-values for the replication (FHS I + FHS II + GOLDN) are provided in Table 2. The direction of association was consistent across both replication cohorts for 47/76 BMI probes [62%, *z* statistic for equal proportions for null hypothesis that each of two cohorts was concordant with ARIC (null = 12.5%, or $0.5 \times 0.5 \times 0.5$); $z = 5.66$, $P = 0.0001$]. Of these 47, meta-analysis *P*-value was < 6.6×10^{-4} for 37 CpG probes. Top probes were those mentioned earlier near *CPT1A* (global meta-analysis *P*-value = 3.4×10^{-47}), near *ABCG1* (global *P*-value = 1.0×10^{-46}), in an intergenic region on Chr 17 (cg03078551, $P = 6 \times 10^{-28}$) and near *SREBF1* (cg11024682, $P = 2.8 \times 10^{-24}$) (sterol regulatory element-binding transcription factor 1), which is intimately involved in cholesterol biosynthesis, producing a product that binds the sterol regulatory element-1 (*SRE-1*), flanking the low density lipoprotein (LDL) receptor gene. Interestingly, the *HIF3A* probes initially reported by Dick et al. (27) to be associated with BMI, and that we confirmed in African Americans earlier, did not replicate in leukocyte DNA from Whites in FHS and GOLDN.

Of the 164 WC-associated probes taken forward for replication in GOLDN, 140 (85%) had consistent direction of association [*z*-statistic for equal proportions for null hypothesis of concordance with ARIC (null = 50%); $z = 3.79$, $P = 0.0001$]. Of these 140, 8 also passed the 3×10^{-4} significance threshold for replication, 1 of which was unique to WC: *DHCR24* (Table 3). *DHCR24* (3-beta-hydroxysterol delta-24-reductase) catalyzes reduction of sterol intermediates during the final step of cholesterol biosynthesis and is of current interest as a biomarker of nonalcoholic hepatic steatosis. *DHCR24* gene expression changes track strongly with weight loss after bariatric surgery (37).

Influence of cis-acting SNPs

As a global test of whether these associations are potentially confounded by any cis-acting single nucleotide polymorphisms (SNPs), we identified SNPs in a 500-kb window (250 kb up and down stream) of each of the 37 replicated BMI probes (see below) and determined their associations with BMI in an existing large GWAS meta-analysis conducted in ~40 000 African American adults (38). Supplementary Material, Table S6 lists the SNP name having the lowest *P*-value for association with BMI in each 500-kb region (referred to as the 'reference SNP'). All such SNPs were associated with BMI at $P = 1 \times 10^{-5}$ or higher. This analysis shows that replicated methylation associations reported here are unlikely to be confounded by the effect of common cis-acting SNPs on BMI in African Americans.

Secondary Analysis 1: impact of prevalent diabetes on replicated associations

Because increased adiposity is a risk factor for type 2 diabetes, and because diabetes and its treatment may impact methylation, we tested the degree to which association results were altered by covariate adjustment for concurrent diabetes status, a possible mediator of the adiposity–DNA methylation relationship. Supplementary Material, Table S7 presents a side-by-side comparison of beta values and *P*-values when diabetes is, and is not, included as a covariate for the 37 replicated BMI probes and the

Table 2. Replication^a of BMI-DNA methylation associations in the FHS (Cohorts I and II) and GOLDN cohorts, in order of meta-analysis P-value

CpG marker	CHR	Closest gene	ARIC (discovery cohort) analysis		Replication meta-analysis (FHS I + FHS II + GOLDN)		Discovery + replication cohort meta-analysis				
			ARIC regression coefficient	ARIC P-value	Replication z statistic	Replication P-value	Direction	Summary P-value	HetISq	HetChiSq	HetPVal
cg00574958	11	CPT1A	-0.0029	3.23E-12	-12.881	5.77E-38	----	3.44E-47	55.9	6.801	0.0785
cg06500161	21	ABCG1	0.0081	1.52E-13	12.456	1.29E-35	++++	1.01E-46	77.8	13.543	0.0036
cg03078551	17	NA	-0.0026	7.49E-09	-9.399	5.52E-21	----	6.03E-28	1.4	3.042	0.3851
cg11024682	17	SREBF1	0.0068	9.58E-09	8.424	3.63E-17	++++	2.76E-24	55.6	6.755	0.0801
cg13123009	6	LY6G6E; LY6G6D	0.0069	1.80E-11	6.894	5.41E-12	++++	1.01E-21	0	1.657	0.6466
cg04927537	17	LGALS3BP	0.0108	5.91E-10	7.17	7.48E-13	++++	2.93E-21	0	2.45	0.4845
cg27243685	21	ABCG1	0.0057	3.61E-08	7.661	1.85E-14	++++	4.23E-21	61.3	7.747	0.0515
cg26403843	5	RNF145	0.0099	1.06E-08	6.934	4.09E-12	++++	2.51E-19	0	0.567	0.9040
cg09349128	22	NA	-0.0059	1.44E-08	-6.92	4.51E-12	----	3.76E-19	0	0.507	0.9173
cg07814318	15	KLF13	0.0081	2.33E-10	5.896	3.73E-09	++++	1.19E-17	73.8	11.445	0.0095
cg07573872	19	SBNO2	-0.0069	2.80E-08	-6.377	1.80E-10	----	3.00E-17	0	0.683	0.8771
cg13708645	12	KDM2B	0.0096	4.68E-10	5.74	9.48E-09	++++	6.03E-17	60.7	7.631	0.0543
cg25178683	17	LGALS3BP	0.0087	1.67E-08	6.138	8.36E-10	++++	9.25E-17	0	0.869	0.8328
cg12992827	3	NA	-0.0075	5.31E-09	-5.939	2.87E-09	----	1.25E-16	47.3	5.693	0.1275
cg09664445	17	KIAA0664	0.0055	8.89E-09	5.93	3.03E-09	++++	2.08E-16	65.4	8.672	0.0340
cg06876354	2	RALB	0.0057	1.02E-09	5.62	1.91E-08	++++	2.53E-16	39	4.916	0.1780
cg23998749	1	NA	0.0052	5.66E-08	5.92	3.22E-09	++++	1.14E-15	40.4	5.035	0.1692
cg06192883	15	MYO5C	0.0065	5.29E-08	5.791	7.01E-09	++++	2.45E-15	0	0.979	0.8062
cg26033520	10	NA	0.0070	1.44E-08	5.578	2.43E-08	++++	3.01E-15	0	1.065	0.7856
cg07136133	11	PRR5L	-0.0048	6.70E-09	-5.36	8.34E-08	----	6.24E-15	0	1.871	0.5996
cg08972190	7	MAD1L1	0.0054	6.43E-10	5.054	4.33E-07	++++	6.42E-15	27.7	4.149	0.2458
cg06946797	16	NA	-0.0070	8.35E-09	-5.131	2.88E-07	----	3.04E-14	17.2	3.622	0.3052
cg18568872	15	ZNF710	0.0062	3.74E-10	4.709	2.49E-06	++++	3.54E-14	66.8	9.04	0.0288
cg15871086	18	NA	0.0062	8.31E-11	4.354	1.34E-05	++++	1.00E-13	67.5	9.226	0.0264
cg08857797	17	VPS25	0.0063	3.40E-09	4.74	2.14E-06	++++	1.55E-13	39.9	4.99	0.1725
cg20954977	2	B3GNT7	0.0101	1.68E-08	4.898	9.66E-07	++++	2.13E-13	21.3	3.811	0.2826
cg04816311	7	C7orf50	0.0090	2.56E-12	3.823	1.32E-04	++++	2.18E-13	77.1	13.094	0.0044
cg14017402	2	NA	0.0080	1.47E-10	4.226	2.38E-05	++++	3.17E-13	75.9	12.462	0.0060
cg17560136	8	EPB49	0.0063	3.03E-08	4.824	1.41E-06	++++	5.27E-13	25.3	4.018	0.2595
cg11592786	15	NA	-0.0031	3.60E-08	-4.584	4.56E-06	----	2.36E-12	14.8	3.52	0.3181
cg01844514	7	ZNF862	0.0046	3.17E-08	4.27	1.96E-05	++++	1.21E-11	4.5	3.143	0.3701
cg18307303	5	IL12B	0.0044	4.67E-08	4.217	2.48E-05	++++	2.16E-11	46.4	5.592	0.1332
cg04869770	1	PBX1	0.0057	5.65E-09	3.805	1.42E-04	++++	4.23E-11	57.6	7.074	0.0696
cg00863378	16	BBS2	0.0055	1.91E-08	3.967	7.27E-05	++++	4.24E-11	49.5	5.939	0.1146
cg26354221	22	ADORA2A	0.0032	1.39E-08	3.725	1.95E-04	++++	1.20E-10	52.7	6.343	0.0961
cg15695155	12	KDM2B	0.0079	4.03E-08	3.802	1.43E-04	++++	1.71E-10	49	5.883	0.1175
cg27614723	15	SLCO3A1	0.0071	5.62E-08	3.62	2.94E-04	++++	5.44E-10	39.4	4.947	0.1757

^aSuccessful replication was defined as consistent direction of association across all four studies (ARIC, FHS I, FHS II and GOLDN) and $P < 0.05/76$ ($< 6.6 \times 10^{-4}$) in the replication meta-analysis. Meta-analysis used Stouffer's Z for trend test, which is based on meta-analysis of P-values with adjustment for cohort sample size and direction.

Table 3. Replication^a of WC-DNA methylation associations in the GOLDN cohort (N = 911), in order of replication P-value

CpG marker	Chr	Nearest gene	ARIC regression coefficient (per 1 SD WC)	ARIC (P-value)	GOLDN regression coefficient (per 1 SD WC)	GOLDN (P-value)	Direction	Combined meta-analysis (P-value)
cg13708645	12	KDM2B	0.0098	7.22E-11	0.0074	1.28E-05	++	4.52E-15
cg11024682	17	SREBF1	0.0080	3.52E-12	0.0039	1.33E-05	++	2.37E-16
cg03078551	17	intergenic	-0.0025	8.16E-09	-0.0021	1.74E-05	--	6.61E-13
cg06192883	15	MYO5C	0.0083	1.50E-12	0.0031	2.56E-05	++	2.15E-16
cg26403843	5	RNF145	0.0101	2.38E-09	0.0044	7.53E-05	++	7.92E-13
cg13123009	6	LY6G6E	0.0074	1.81E-13	0.0032	8.25E-05	++	1.07E-16
cg06500161	21	ABCG1	0.0096	4.41E-19	0.0043	9.39E-05	++	1.09E-21
cg17901584	1	DHCR24	-0.0080	8.34E-08	-0.0049	2.39E-04	--	8.15E-11

^aSuccessful replication was defined as consistent direction of association in GOLDN and ARIC and P-value of $< 0.05/164$ ($< 3 \times 10^{-6}$) in the replication cohort. Meta-analysis used Stouffer's Z for trend test, which is based on meta-analysis of P-values with adjustment for cohort sample size and direction.

1 additional replicated WC probe (above). Generally, regression coefficients were reduced by 5–25% with adjustment, but 19/37 of BMI-associated probes and 0/1 of the WC probes remained significant at $P < 1 \times 10^{-7}$. These results show that about half of the BMI–DNA methylation associations we report are independent of concurrent diabetes, and the others remain strongly associated even with adjustment for this condition.

Secondary Analysis 2: replication in adipose tissue samples

The 37 BMI probes and 1 WC methylation probe that independently replicated in FHS and GOLDN were subjected to cross-tissue association study using adipose tissue DNA (Table 4). Replication was based on P -value only, because it was assumed that the direction of effect of the same exposure on DNA methylation may differ in different tissue types. Twenty-eight of the 38 probes passed quality control (QC) in Multiple Tissue Human Expression Resource (MuTHER), and of these, 18/28 (64%) were associated with BMI in adipose tissue at $P < 1.9 \times 10^{-3}$, including markers near the previously identified *CPT1A* and *ABCG1* loci, but also a large number of novel adiposity-related loci, including *LYS6GE*, *KDM2B*, *RALB*, *PRRL5*, *LGALS3BP*, *C7orf50*, *PBX1*, *EPB49* and *BBS2*. *BBS2* [Bardet–Biedl syndrome (BBS) 2] is one of the BBS genes involved in cilia formation, cell movement and cell signaling. Autosomal recessive variants in *BBS2* are among those responsible for BBS, which is characterized by severe obesity and numerous developmental aberrations. Overall, the results show wide cross-tissue agreement in BMI- and WC-methylation associations.

Discussion

Our study presents the first EWAS of adiposity traits in African American adults, demonstrating numerous methylation variants associated with BMI and WC, including 37 CpG site associations for BMI and 1 additional association for WC that replicated in two European-ancestry cohorts. The ARIC results included sites in three loci (near *HIF3A*, *CPT1A* and *ABCG1*) that have been previously reported to be associated with BMI, insulin-related traits and lipid subfractions (27,33,41,42), as well as a number of novel loci. This demonstrates to our knowledge the first cross-ethnic replication of methylation signals for BMI from the HM450K array. Further, the results show that despite differences in blood cell type (GOLDN used CD4+ T cells, ARIC and FHS used whole blood), normalization approach [GOLDN used COMBAT followed by polynomial regression normalization (43), FHS used watermelon and Beta Mixture Quantile dilation (BMIQ) (44) and ARIC used BMIQ] and some variation in the average age of the cohorts and specific covariate adjustments, there was general consistency of results across these large, independent EWAS studies of adiposity traits. This is important because while epigenetic modification in cancer etiology has been established for more than a decade (45,46), identification of epigenetic patterns involved in cardiometabolic disease and its precursors (e.g. obesity) has yielded relatively few replicated loci (reviewed by Drong *et al.*) (7). There has been concern that the large potential for confounding in epigenetic studies would make successful replication difficult. What is likely more important to successful replication of adiposity associations is sample size; effect sizes observed in this study were relatively small, with the marginal variance in methylation beta value at individual CpG sites explained by a 1 SD difference in BMI or WC ranging from 0.25 to 2.4%.

ARIC participants gained an average of 7 kg/m² over the 30-year period from age 25 to Visit 2. BMI change was associated

with eight different CpG sites, including the highly cited methylation variant near *CPT1A*, showing that numerous novel methylation loci identified in our study do not only index current weight status or its correlates but may also be involved in changes in body weight over time. Generally, we observed extensive overlap in the methylation associations for the three adiposity traits investigated; of the 38 replicated CpG associations for BMI/WC, 4 were shared by all 3 traits, and 27 were shared by BMI and WC (see Fig. 3 for a diagram of overlap across the 3 traits).

Significant obesity or weight gain/weight loss associations have been reported for CpG methylation sites near *SLC6A4* (47), *MEST* (48), *NPY* (49), *POMC* (49), *PGC-1 α* and *PDK4* (50). Existing studies tend to be small (generally <200 subjects per group), which likely explains the lack of replication. Exceptions include replicated methylation associations near *POMC* (20) and *RXRA* (21). None of these CpG sites from previously reported regional or candidate gene association studies was significantly associated with BMI, WC or BMI change in the present analysis. We also did not replicate the methylation variants recently found to change in response to weight loss (51) or increased physical activity (52). A recent genome-wide BMI methylation study conducted in 48 obese and 48 lean African American children did not report associations at the CpG site level but indicated that differentially methylated regions with greater inter-individual variance in methylation are enriched in obesity, as in cancer (25).

To date, the only prior EWAS to identify and robustly replicate methylation variants associated with BMI is that of Dick *et al.* (27). That study reported methylation at three CpG sites in intron 1 of *HIF3A* to be positively associated with BMI in both blood and adipose tissue DNA in European adults (27). Here, we replicate the positive association for three of these probes in blood DNA from African Americans (cg22891070, cg16672562 and cg27146050) with BMI and between cg16672562 methylation and BMI change. As described previously by Dick *et al.* (27), these probes are within likely regulatory elements (open chromatin regions) and are potentially functional; moreover, methylation level was inversely associated with *HIF3A* gene expression at one of the five expression probes examined. *HIF3A* is a component of the hypoxia-inducible transcription factor (HIF) involved in the physiological response to hypoxia but is also implicated in adipocyte differentiation (53) and is expressed in response to glucose and insulin changes (54). However, we found that these *HIF3A* methylation probes were not associated with BMI in either the FHS or the GOLDN study. The reason for this inconsistent replication is unclear but may relate to the greater obesity comorbidities (perhaps including inflammatory status) in ARIC (e.g. diabetes) and the original study population (over 50% of whom had a history of myocardial infarction), when compared with the replication cohorts in this analysis.

Our unbiased pathway analysis of the EWAS data shows that over 100 biological pathways are significantly enriched for methylation association with BMI and WC, including those involved in lipid and energy metabolism, immune function, adipocyte, neuronal and chondrocyte differentiation and development, and many others. These results suggest that further work in larger cohort studies may identify many additional methylation variants in association with adiposity and its related traits. Many of the specific loci identified in this study are known to be involved in lipid and lipoprotein metabolism, including the known differentially methylated locus *CPT1A* (involved in mitochondrial uptake of long-chain fatty acids and triglyceride metabolism) and *ABCG1* (involved in macrophage cholesterol and phospholipids transport, and lipid homeostasis), as well as the novel BMI-related methylation site near *SREBF1*, which gene

Table 4. Replicated BMI CpG sites significantly associated in both leukocyte and adipose tissue DNA from 648 females in the MuTHER, in order of MuTHER P-value^a

CpG marker	Nearest gene	MuTHER regression coefficient ^b	Same direction as in blood?	MuTHER P-value ^c	Function
BMI					
cg17560136	EPB49	0.00233	Yes	4.64E – 33	Erythrocyte membrane protein band 4.9: gene product essential for the maintenance of erythrocyte shape and membrane stability
cg04869770	PBX1	0.00222	Yes	8.53E – 25	Pre-B-cell leukemia transcription factor involved in pancreatic development and function, candidate gene SNPs associated with obesity, variants may influence porcine adipose tissue fatty acid composition
cg25178683	LGALS3BP	0.00305	Yes	1.68E – 18	Lectin, galactoside-binding, soluble, 3 binding protein: a macrophage inflammatory marker, arterial expression upregulated in response to obesity in animal models
cg01844514	ZNF862	0.00111	Yes	4.72E – 16	Zinc finger protein 862: protein coding gene of unknown function, may be involved in transcriptional regulation
cg07136133	PRR5L	–0.00392	Yes	7.85E – 15	Proline rich 5-like: regulates cytoskeleton organization and interacts with mTOR, a central controller of cell growth, to increase apoptosis
cg06876354	RALB	–0.00129	No	9.40E – 14	RAS-related small GTP-ase B: involved in a variety of cellular processes including gene expression and has role in colorectal cancer oncogenesis
cg26033520	Intergenic Chr. 10	0.00244	Yes	5.29E – 12	Unknown
cg08857797	VPS25	–0.00172	No	5.85E – 12	Vacuolar protein-sorting-associated protein 25: part of endosomal sorting complexes required for transport protein, II (ESCRT-II) complex involved in endosomal transport and possibly gene transcription
cg06946797	Intergenic Chr. 16	–0.00145	Yes	2.09E – 09	Unknown
cg04816311	C7orf50	–0.00152	No	2.27E – 09	Chromosome 7 open reading frame 50; unknown function, GWAS variants in this locus are associated with lipid levels and longevity
cg00574958	CPT1A	–0.00032	Yes	3.40E – 08	Carnitine palmitoyltransferase-1A: involved in mitochondrial uptake of long-chain fatty acids and triglyceride metabolism, previously associated with insulin-related DNA methylation
cg13123009	LY6G6E	0.00072	Yes	1.18E – 07	Lymphocyte antigen-6 complex, locus G6E (pseudogene): one of LY6 genes located in the MHC class III region, immune function, acute lymphoblastic leukemia, hematopoiesis, cell adhesion
cg13708645	KDM2B	0.00184	Yes	1.74E – 07	Lysine K-specific demethylase 2B: a H3K36 histone demethylase, involved in cellular senescence, tumor cell differentiation, part of a complex that represses preadipocyte differentiation
cg26403843	RNF145	–0.00203	Yes	5.59E – 07	Ring finger protein 145: unknown function
cg00863378	BBS2	0.00113	Yes	7.21E – 07	BBS-2: member of the BBS gene family. BBS is an autosomal recessive disorder characterized by severe obesity, developmental abnormalities and mental retardation
cg09664445	KIAA0664	0.00077	Yes	9.84E – 06	Aka CLUH, clustered mitochondria homolog: regulates mitochondrial biogenesis
cg18568872	ZNF710	0.00094	Yes	1.85E – 05	Zinc finger 710: protein-coding gene, function unknown, may be involved in transcriptional regulation
cg15871086	Intergenic Chr. 10	0.00094	Yes	1.46E – 03	Unknown
WC					
cg17901584	DHCR24	0.00162	No	9.07E – 13	3-Beta-hydroxysterol delta-24-reductase: catalyzes reduction of sterol intermediates during final step of cholesterol biosynthesis, biomarker of nonalcoholic hepatic steatosis, expression changes associated with weight loss after bariatric surgery

^aThe MuTHER includes genome-wide DNA methylation data (Illumina HumanMethylation450 array) from abdominal subcutaneous adipose tissue collected from 648 female twins (39,40).^bFixed-effects regression coefficient from linear mixed models (LMM), with probe DNA methylation beta value as the dependent variable and BMI as the primary independent variable, covariate adjusted for age, bisulfite conversion concentration, bisulfite conversion efficiency and experimental batch (Beadchip) (fixed effects), and family relationship (twin-pairing) and zygosity (random effects).^cReplication was based on P-value only because increased or decreased methylation may differ by tissue type. Threshold for replication was set at $P < 1.8 \times 10^{-3}$ (0.05/28 tests). There were 37 BMI-associated CpGs and 1 WC-associated CpG carried forward for replication in adipose tissue, and 10 were removed in QC procedures in MuTHER cohort and not tested.

CpGs shown replicated successfully in blood DNA in GOLDN (WC) or GOLDN and FHS (BMI)
 ** = CpGs also significantly associated with BMI using adipose tissue DNA in MuTHER
Underlined = Independent of T2DM status

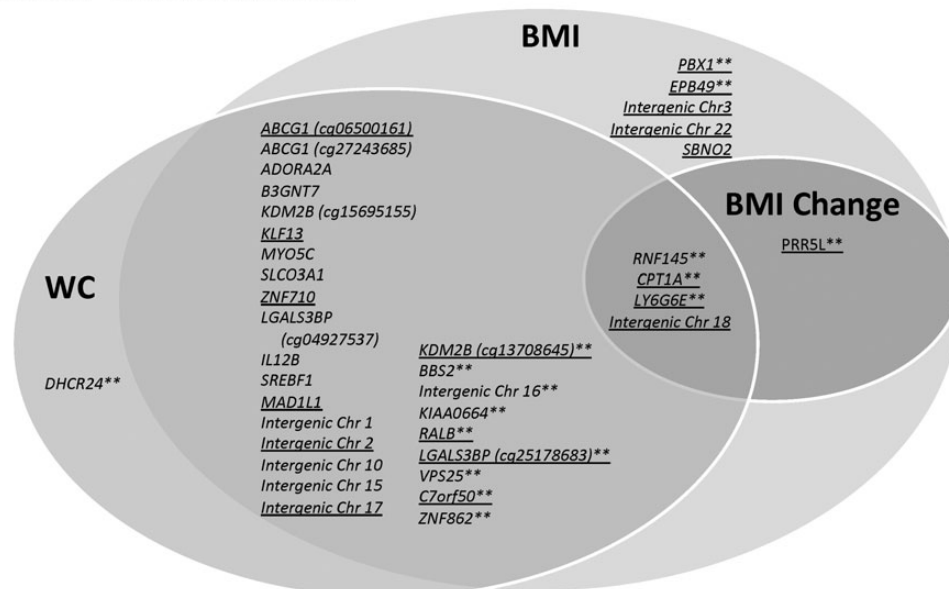


Figure 3. Overlap in EWAS results for BMI, WC and BMI change in ARIC African American adults.

encodes a transcription factor that binds to the LDL receptor and other genes involved in sterol biosynthesis. Promoter variants in *SREBF1* influence hepatic cholesterol and steatosis in rats (55) and with diabetes traits in humans (56,57). Immune function and inflammatory pathways are also strongly represented in our novel loci, including *IL-12B*, *NFKBIL1*, *LY6G6E*, *SBNO2* (part of the *IL-10* anti-inflammatory signaling pathway) and *LGALS3BP* (lectin galactoside-binding soluble 3 binding protein). *LGALS3BP* expression was recently found to be one of a small group of genes whose expression is strongly modulated throughout the arterial network in response to obesity (58). This study together with our findings suggests how obesity-related methylation variants could be useful as targets for cardiovascular disease treatment and prevention. To move this work forward, the functional significance of the methylation variants discovered here must be established and a determination made that they mediate the relationships of obesity to diabetes and CHD, rather than that they are downstream effects of the obesity-related disease.

In that regard, we were interested in whether the associations identified with adiposity traits were likely mediated by or otherwise dependent on the presence of diabetes, which condition was common in our study sample (26%), is strongly related to obesity and is associated with methylation variation (e.g. 26,59). Although in half of the sites, *P*-values were no longer genome-wide significant after covariate adjustment for prevalent diabetes, all of the associations remained strong ($P < 1 \times 10^{-5}$), with an average change in regression coefficient of only 11.2%. Thus, while some adiposity-related methylation signals may be due to underlying insulin resistance or diabetes, these results suggest that many may be properly characterized as fundamentally obesity related. Longitudinal analysis of weight gain in individuals without diabetes will confirm these findings.

Blood is an accessible and plentiful tissue for genomic analysis in large studies, but there are two major concerns regarding its use in epigenetic studies of obesity and cardiometabolic conditions: biological relevance and confounding by cell type

differential. In terms of relevance, obesity may be characterized as a defect in appetite/satiety regulation resulting in elevated circulating free fatty acids, leading to adipocyte differentiation to sequester the excess lipids. The most biologically relevant tissue types for the analysis of obesity-related gene expression (and, ergo, methylation) might therefore include the hypothalamus, liver and adipose tissue. It is encouraging that many of the signals we detected in WBCs also replicated in adipose tissue. Not all did so, perhaps due to gender differences: MuTHER is 100% female while the studies using blood included both males and females. It has been shown that different tissues including brain, lung, thyroid, saliva and whole blood exhibit highly concordant age associations (60) and that smoking is associated with consistent *AHRR* methylation profiles in both lymphoblasts and pulmonary macrophages (61). Such studies suggest that DNA methylation in blood can serve as a biomarker of methylation in other tissues, as found here.

Another concern regarding the use of blood in epigenetic epidemiologic investigations is that it contains multiple cell types, each having a characteristic methylation profile, such that DNA methylation associations with disease could be confounded by the relative proportions of different cell types in the DNA sample (62). Indeed, mean methylation percent differed significantly between blood cell types for over 70 000 of the CpG sites on the HM450 (63). This was less of a concern here in that there was no association between WBC differential and obesity at Visit 1 in ARIC (when measured differentials were available for the entire cohort). In addition, covariate adjustment for imputed WBC differentials did not substantially alter our EWAS results, and we replicated our results in different tissue/cell types, both of which procedures reduce the probability that the results are solely due to confounding by cell type distribution.

Despite its disadvantages, an advantage of leukocytes for epigenetic studies of obesity is that adiposity and immunological activation are strongly causally related. When individuals gain excessive body fat, numerous changes in the immune system

occur, including increases in WBCs and alterations in the production of different leukocytes, including increases in neutrophils, mast cells, CD8+ T cells and some classes of monocytes and decreases in T regulatory cells and eosinophils. These changes in immune cell distribution reduce the production of anti-inflammatory IL-10 and increase the production of pro-inflammatory IL-6, IFN γ and tumor necrosis factor alpha (TNF α), among other cytokine changes (64). After substantial weight loss, leukocyte counts and differentials normalize, as seen following bariatric surgery (65), which suggests that weight gain and loss drive changes in low-grade inflammation and leukocyte cell types rather than the reverse. Thus, methylation signals found in blood may be potentially useful biomarkers of obesity-related inflammatory damage, as indicated by the many inflammation-related loci identified in the current analysis.

The present study has a number of limitations, the most important of which is the cross-sectional design, which does not allow for clear temporal relationships between predictor (e.g. BMI) and outcome (CpG-specific methylation) to be assessed and leaves open the potential for reverse causality. Studies including non-obese individuals with DNA methylation assessment at that time point with follow-up for incident obesity and DNA methylation changes at a later point would provide clarification on methylation variants that drive the development of obesity as opposed to those that are affected by obesity. Second, gene expression data were not available in ARIC to confirm the functional relevance of the DNA methylation variation we have identified. However, the *CPT1A*, *HIF3A* and *ABCG1* probes we report have been found to influence gene expression in prior studies (27,41).

The study also had numerous strengths, including a relatively large discovery sample, which may have been responsible for the larger number of significant findings than has been reported previously for BMI, a focus on African Americans, which is important due to their higher burden of obesity and related conditions but poorer coverage by current EWAS studies, and control for numerous potential confounders and batch effects, including a test of potentially cis-acting SNPs in each of the methylation regions for association with BMI, and covariate adjustment for imputed cell type differentials. Other strengths include examination of multiple adiposity traits, a relatively large replication sample ($N = 3368$) with additional replication in adipose tissue as well ($N = 648$), quantification of probe-specific measurement error/reliability and consideration of the role of diabetes in the associations.

In conclusion, this study confirmed three previously identified methylation loci suggested to be associated with obesity and related traits (*CPT1A*, *ABCG1* and *HIF3A*) and identified numerous additional novel loci harboring individual DNA methylation variation in both blood and adipose tissue that are associated with adiposity traits in African American adults. Results were successfully replicated across studies despite variation in tissue type, ethnicity and analytic approaches. Experimental and longitudinal study designs, and larger multi-cohort analyses, are needed to assess causality and to move the growing field of epigenetic epidemiology toward richer insight into the biology of obesity, as well as new therapies to reduce or reverse its downstream effects on health.

Methods and Procedures

Population

The ARIC Study is a prospective cohort study of cardiovascular disease risk in four US communities (31). Between 1987 and

1989, 7082 men and 8710 women aged 45–64 years were recruited from Forsyth County, North Carolina; Jackson, Mississippi (African Americans only); suburban Minneapolis, Minnesota; and Washington County, Maryland. The ARIC Study protocol was approved by the institutional review board of each participating university. After written informed consent was obtained, including that for genetic studies, participants underwent a baseline clinical examination (Visit 1) and four subsequent follow-up clinical exams (Visits 2–5). At this time, DNA methylation data are available for African American members of the cohort only, and the present study comprises a cross-sectional analysis of these data. Specifically, a single DNA sample was chosen for methylation analysis for each subject, and BMI, WC and covariate data detailed below were from the same study visit. For these analyses, all data come from Visit 2, except as noted below for physical activity. In addition, self-reported weight at age 25 (collected only at Visit 1) was used to calculate weight change from age 25 to Visit 2 as a measure of adulthood BMI change.

Measurements and questionnaires

Anthropometrics were taken with the subject wearing a scrub suit and no shoes. BMI was calculated from measured weight and height (weight in kilograms/height in meters squared). WC was measured at the level of the umbilicus using a flexible tape. WBC count was assessed by automated particle counters within 24 h after venipuncture in the local hospital hematology laboratory. The reliability coefficient for the WBC count measurement was >0.96 (66). Measured WBC differentials were only available for a subset at Visit 2 ($N = 187$), but these were used in the imputation of differential WBCs for the remaining subjects (see below for description). Questionnaires assessed education (coded as less than high school degree, high school degree or equivalent and greater than high school degree), current household income, current cigarette smoking (coded as current, former and never smoked), current alcohol consumption status (coded as current/former/never) and medical history (67). Level of leisure time physical activity was assessed at Visit 1 using the Baecke questionnaire (68). Leisure time activity scores range in whole and half increments from 1 to 5, with values <2 indicative of physical inactivity (69). Prevalent diabetes was defined as a fasting glucose level of ≥ 126 mg/dl (70), nonfasting glucose of ≥ 200 mg/dl or a self-reported physician diagnosis of or treatment for diabetes.

Bisulfite conversion of DNA

Genomic DNA was extracted from peripheral blood leukocyte samples using the Genra Puregene Blood Kit (Qiagen; Valencia, CA, USA) according to the manufacturer's instructions (www.qiagen.com). Bisulfite conversion of 1 μ g genomic DNA was performed using the EZ-96 DNA Methylation Kit (Deep Well Format) (Zymo Research; Irvine, CA, USA) according to the manufacturer's instructions (www.zymoresearch.com). Bisulfite conversion efficiency was determined by PCR amplification of the converted DNA before proceeding with methylation analyses on the Illumina platform using Zymo Research's Universal Methylated Human DNA Standard and Control Primers.

Illumina Infinium methylation assay

The Illumina Infinium HumanMethylation450K Beadchip array (HM450K) (described by Sandoval et al.) (71) was used to measure DNA methylation (Illumina, Inc.; San Diego, CA, USA). The

platform detects methylation status of 473 788 CpG sites by sequencing-based genotyping of bisulfite-treated DNA. Bisulfite treatment converts only unmethylated cytosines to uracils, allowing for highly multiplexed genotyping with single site resolution. The array covers 96% of CpG Islands (as well as CpG shores) and 98.9% of RefSeq genes with a global average of 17.2 probes per gene region and has been shown to have high accuracy and reliability (72,73).

Bisulfite-converted DNA was used for hybridization on the HM450K BeadChip, following the Illumina Infinium HD Methylation protocol (www.illumina.com). This consisted of a whole genome amplification step followed by enzymatic end-point fragmentation, precipitation and re-suspension. The re-suspended samples were hybridized to the complete set of bead-bound probes, followed by ligation and single-base extension during which a fluorescently labeled nucleotide is incorporated and scanned. The degree of methylation is determined for each CpG cytosine by measuring the amount of incorporated label for each probe. The intensities of the images were extracted using Illumina GenomeStudio 2011.1, Methylation module 1.9.0 software. The methylation score for each CpG was represented as a beta (β) value according to the fluorescent intensity ratio. Beta values may take any value between 0 (nonmethylated) and 1 (completely methylated). Background subtraction was conducted with the GenomeStudio software using built-in negative control bead types on the array.

Normalization

The HM450K uses two different probe types (I and II). Due to differences in design, probes using the Illumina Type II assay are less sensitive for the detection of extreme methylation values (i.e. 0 and 1) than the Type I assay and have greater average variance between technical replicates (74). BMIQ (75) was used in this analysis to adjust the beta values of type 2 design probes into a statistical distribution characteristic of type 1 probes. BMIQ has been shown to more effectively reduce probe set bias and technical error across replicates compared with some other peak-based and quantitative normalization procedures (76). An emerging conclusion is that in general, the improvements offered by different normalization approaches are modest, with very high concordance in association results across different methods (77). Further, in this study, we conducted all analyses at the single probe level, and therefore, any differences in probe type should not strongly influence the results.

Quality control

Positive and negative controls and sample replicates were included on each 96-well plate assayed. After exclusion of controls, replicates and samples with integrity issues or failed bisulfite conversion, a total of 2841 study participants had HM450K data available for further QC analyses. We removed poor-quality samples with pass rate of <99%, that is, if the sample had at least 1% of CpG sites with detection P -value > 0.01 or missing ($N = 37$), indicative of lower DNA quality or incomplete bisulfite conversion, and samples with a possible gender mismatch based on evaluation of selected CpG sites on the Y chromosome ($N = 2$), leaving a total of 2802 samples available for analysis. At the target level, we flagged poor-quality CpG sites with average detection P -value of >0.01 and calculated the percentage of samples having detection P -value of >0.01 for each autosomal and X chromosome CpG site. There were 9399 autosomal and X chromosomal markers where

>1% of samples showed detection P -value of >0.01, and these sites were excluded. In addition, we filtered 370 CpG sites on the Y chromosome with average detection P -value of >0.01, leaving a total of 473 788 CpG sites for analysis.

Technical error analysis

To obtain a measure of probe-specific technical error and the reliability of the methylation measures, technical replicates were included for 130 samples (total $n = 265$ with 5 samples replicated 3 times), from which ICC coefficients for methylation beta values were calculated for all probes (78). Further information on the method is provided in the Supplementary Text.

Statistical analysis

Of the 2802 samples available after methylation QC, 705 did not have complete covariate data needed for confounder adjustment, leaving a final sample size of $N = 2097$ (2096 with BMI and BMI change data and 2097 with WC data). Mean, standard deviation and range, or frequencies, are provided to describe continuously distributed and categorical variables, respectively. Methylation data were tested and reported in terms of beta values, ranging from 0 to 100%. While beta values have non-constant variance across different CpG sites, they have the clear advantage of representing the percentage methylation for each site and are therefore more easily interpretable than M values, which are the log₂ ratio of the intensities of the methylated versus unmethylated probes. Further, it has been shown that in large sample sizes as in ARIC, test statistics are similar for M and beta values (79).

Pathway analysis

Complete methylation and phenotype data were first subjected to pathway analysis for an a priori test of genomic pathway enrichment for the association of BMI or WC with DNA methylation. The Illumina 450 K probe annotation file contains (non-unique) mappings of 75% of the CpG sites to 21 160 genes, based upon the closest gene to each methylation probe. Reflecting our purpose in detecting entire genomic pathways that are enriched for individually small methylation signals, and long-standing practice in pathway analysis of GWAS SNP data, gene-level methylation signal measurements were first summarized by averaging across all probes annotated to each gene (80). Bioconductor was used to assign genes to the GO domains (molecular function, cellular component and biological process), for a total of 6700 GO pathways tested. Pathway enrichment methods that are not resampling based have been shown to be highly anti-conservative (81). We performed pathway testing while rigorously controlling false positives by using the *safeExpress* R package (82), controlling for the same covariates as specified below. We used the *safeExpress* test statistic D , which is a competitive statistic contrasting genes in each pathway versus the complement (82). For each GO domain, the output provided pathway global statistics and P -values, where correction for multiple comparisons was performed using Benjamini–Hochberg FDR q -values (83). The FDR is relatively robust to positive correlation structures (84), which are often strong in pathway analysis and thus enables effective multiple test correction in a manner that is not overly conservative. FDR $q < 0.05$ was considered statistically significant.

Association study

Batch effect adjustment is critical in the analysis of HM450K data, and ComBat is an Empirical Bayes method frequently used to

adjust gene expression and other microarray data for potential batch effects (85). In preliminary analyses, we found very high concordance in EWAS results for BMI with and without ComBat adjustment of the beta values in our linear mixed-effect regression models (LMMs) to address batch effects, where batch effect was accounted for by adding plate number (1–34) and chip row number (1–6) as fixed effects and chip number (1–244) as a random effect. Therefore, we used the simpler LMM without ComBat adjustment for subsequent analyses. We specified the regression models with probe methylation beta value as the dependent variable, and with adiposity traits and all covariates as the independent variables. We chose this approach because the technical (e.g. batch) effects we wished to adjust for pertain to the beta values, not to BMI, and also because in our study of older African American adults, weight gain and obesity are long-standing characteristics of the participants over their lives, which we posit may have influences on DNA methylation (rather than being influenced by DNA methylation).

Cross-sectional LMM were tested using the R package *lme4* with methylation beta values as the dependent variable, and with chip specified as a random effect and the following variables specified as fixed effects: standardized adiposity variable (BMI, WC and BMI change with mean = 0 and SD = 1), sex, age, study center, total WBC, education, household income, current cigarette smoking, current alcohol consumption, leisure time physical activity and 10 PCs from the Illumina Infinium HumanExome Beadchip genotype array (86), to account for potential confounding by genetic ancestry. The regression models were further adjusted for leukocyte cell type proportions (neutrophils, lymphocytes, monocytes and eosinophils) as additional fixed effects. Cell-type proportions were imputed using the algorithm developed by Houseman (40), which utilizes the known cell type specificity of methylation at selected CpG probes on the HM450 to impute cell type proportions, and based on the measured differential cell counts available for a subset of ARIC participants at Visit 2. The choice of covariates was based upon known or suspected confounding, as described in the directed acyclic graph presented in Supplementary Material, Figure 1S. The Wald test was applied to test the hypothesis that BMI, WC or BMI change (depending on the model tested) was associated with CpG methylation.

Following standard practice in association analysis (87), multiple test corrections were used to control the family-wise error at 0.05. Applying a standard Bonferroni correction for the 473 788 CpG sites gives $P < 1 \times 10^{-7}$ as the significance threshold. Significant EWAS results for each trait were then filtered to remove known cross-reactive probes and polymorphic CpGs (88).

A secondary analysis addressed whether the adiposity-methylation associations were independent of diabetes by including prevalent diabetes status (yes/no) in the above regression model. To account for multiple comparisons, a Bonferroni-corrected P -value of $< 1 \times 10^{-7}$ was used as the threshold for determining whether CpG methylation remained significantly associated with adiposity independent of diabetes status.

Replication analysis

CpG sites with association P -values of $< 1 \times 10^{-7}$ for BMI and WC were carried forward for replication in 991 members of the GOLDN cohort (mean age = 49 years, mean BMI = 28 kg/m², female = 52%, prevalent diabetes = 7.6%, ancestry = 100% European-American, tissue = CD4 + T cells) and 2377 members of the FHS Offspring (mean age = 67 years, mean BMI = 28 kg/m², % female = 56%, prevalent diabetes = 13.4%, ancestry = 100%

European-American, tissue = whole blood). Both studies utilized blood DNA and the same HM450 platform for methylation analysis as in ARIC. Methylation assays were performed at two different laboratories in the FHS and thus are considered as two independent cohorts for replication. Further details of the two replication cohorts are provided in the Supplementary Text. Given the different analytic strategies and blood cell types used among the discovery and replication studies (detailed in the Supplementary Text), the meta-analysis was conducted on P -values (not beta values) using a sample size-weighted method (Stouffer's Z trend) that also incorporated the direction of the beta coefficients (89) and was implemented in R. For BMI, replication in blood DNA was defined as consistent direction of the beta coefficient in all four cohorts, and a Bonferroni-corrected meta-analysis P -value for the replication cohorts (FHS I, FHS II and GOLDN) of $< 0.05/76$ or $P < 6.6 \times 10^{-4}$. For WC, replication was only available in the GOLDN cohort, and probe associations were considered to have positively replicated based on consistency of the direction of effect and a Bonferroni-corrected P -value based on the number of probes tested (164 probes, $0.05/164 = P < 3 \times 10^{-4}$).

Test for confounding by cis-acting genetic variants

Methylation SNPs, defined as SNPs within probe sequences, were filtered as stated earlier. As a more general means of excluding the possibility that our results are confounded by cis-acting SNPs, we examined the 500-kb regions surrounding each of our top BMI-associated methylation probes to search for SNP associations with BMI using data from a recent large meta-analysis and report the lowest P -values for each region. This meta-analysis examined the association of > 3.2 million SNPs (imputed using the 1000 Genomes Project) with BMI in 39 144 men and women of African ancestry (38).

Cross-tissue replication

Concerns regarding tissue specificity of CpG site methylation and the relevance of peripheral blood leukocytes for obesity were addressed in a secondary analysis by testing the association of the replicated CpG probes identified for BMI ($N = 37$) and WC ($N = 1$) using adipose tissue DNA. The MuTHER contains genome-wide DNA methylation data using the HM450 array in subcutaneous abdominal adipose tissue collected from 648 European-ancestry female twins and singletons (97 MZ pairs, 162 DZ pairs and 130 singletons) (39,40). The subjects had a mean age of ~60 years and a mean BMI of 26.6 kg/m². Further details on the MuTHER cohort materials and methods are found in the Supplementary Text. The threshold for replication was set at Bonferroni-corrected $P < 1.9 \times 10^{-3}$ (0.05/28 tests). There were 38 BMI- or WC-associated CpGs carried forward for replication in adipose tissue, and 10 were removed in QC procedures in MuTHER cohort and not tested.

Supplementary Material

Supplementary Material is available at HMG online.

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