CPT1A methylation is associated with plasma adiponectin

S. Aslibekyan [a,*], A.N. Do [a], H. Xu [c], S. Li [d], M.R. Irvin [a], D. Zhi [b], H.K. Tiwari [b], D.M. Absher [f], A.R. Shuldiner [c], T. Zhang [d], W. Chen [d], K. Tanner [c], C. Hong [c], B.D. Mitchell [c,e], G. Berenson [d], D.K. Arnett [a,1]

[a] Department of Epidemiology, University of Alabama at Birmingham, USA
[b] Department of Biostatistics, University of Alabama at Birmingham, USA
[c] Department of Medicine, University of Maryland School of Medicine, USA
[d] Department of Epidemiology, School of Public Health and Tropical Medicine, Tulane University, USA
[e] Geriatrics Research and Education Clinical Center, Baltimore Veterans Administration Medical Center, Baltimore, MD, USA
[f] HudsonAlpha Institute for Biotechnology, USA

Abstract *Background and aims:* Adiponectin, an adipose-secreted protein that has been linked to insulin sensitivity, plasma lipids, and inflammatory patterns, is an established biomarker for metabolic health. Despite clinical relevance and high heritability, the determinants of plasma adiponectin levels remain poorly understood.

Methods and results: We conducted the first epigenome-wide cross-sectional study of adiponectin levels using methylation data on 368,051 cytosine-phosphate-guanine (CpG) sites in CD4+ T-cells from the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN, n = 991). We fit linear mixed models, adjusting for age, sex, study site, T-cell purity, and family. We have identified a positive association (regression coefficient ± SE = 0.01 ± 0.001, P = 3.4 × 10^{-13}) between plasma adiponectin levels and methylation of a CpG site in *CPT1A*, a key player in fatty acid metabolism. The association was replicated (n = 474, P = 0.0009) in whole blood samples from the Amish participants of the Heredity and Phenotype Intervention (HAPI) Heart Study as well as White (n = 592, P = 0.0005) but not Black (n = 243, P = 0.18) participants of the Bogalusa Heart Study (BHS). The association remained significant upon adjusting for BMI and smoking in GOLDN and HAPI but not BHS. We also identified associations between methylation loci in *RNF145* and *UFM1* and plasma adiponectin in GOLDN and White BHS participants, although the association was not robust to adjustment for BMI or smoking.

Conclusion: We have identified and replicated associations between several biologically plausible loci and plasma adiponectin. These findings support the importance of epigenetic processes in metabolic traits, laying the groundwork for future translational applications.

* Corresponding author. University of Alabama at Birmingham, 1665 University Blvd, Ryals Public Health Building, 230J, Birmingham, AL 35294, USA. Fax: +1 205 934 8665.
E-mail addresses: saslibek@uab.edu, stella7@gmail.com (S. Aslibekyan).
[1] Present address: School of Public Health, University of Kentucky, Louisville, KY, USA.

Introduction

Adiponectin is an adipocyte-derived secretory protein with generally positive, multi-pronged effects on metabolic health [1]. Levels of circulating adiponectin in the plasma are heritable (h^2 = 55.1%) [2] and widely used as a biomarker for cardiovascular and metabolic risk. Despite

clinical relevance and high heritability, the genetic determinants of plasma adiponectin remain poorly understood, with the known genetic polymorphisms accounting for only a modest fraction of inter-individual variability [3]. An emerging body of evidence suggests that epigenetic changes such as DNA methylation may contribute to the "missing heritability" and merit further evaluation as risk factors for metabolic disease [4]. However, prior epigenetic investigations into the adiponectin pathway were largely limited in scope and/or sample size, focusing on candidate regions rather than epigenome-wide variation. The advent of high-throughput array-based methods has enabled a comprehensive approach to quantifying epigenetic variation, promising new insights into the relationship between DNA methylation and complex traits such as circulating adiponectin.

Using DNA isolated from CD4+ T-cells from 991 European American participants of the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN), we have performed the first cross-sectional epigenome-wide study (EWAS) of plasma adiponectin. We subsequently replicated our top finding in two external populations, using whole blood samples from the Heredity and Phenotype Intervention (HAPI) Heart Study (n = 474) and the Bogalusa Heart Study (BHS, n = 835). We further conducted sensitivity analyses to account for the potential role of obesity, smoking, and alcohol in the relationship between DNA methylation and circulating adiponectin. Finally, we have examined methylation variation in regions reported to be associated with circulating adiponectin in prior genetic studies to provide a more comprehensive view of heritable determinants of cardiometabolic health.

Methods

Study populations

Discovery
The GOLDN study population, previously described in detail [5–8], was comprised of European American families with at least two siblings, recruited from the participants of the National Heart, Lung, and Blood Institute Family Heart Study in Minneapolis and Salt Lake City. Before each study visit, participants abstained from eating for 8 h, drinking alcohol for 24 h, and using other lipid-lowering medications for 4 weeks. The Institutional Review Boards at the University of Minnesota, University of Utah, and Tufts University/New England Medical Center approved the study protocol, and all participants provided informed consent.

Replication
BHS is a long-term community-based study of cardiovascular risk factors in a biracial (Black/White) rural community in Louisiana [11]. Participants provided informed consent, while the study design and procedures as well as consent forms were approved by Institutional Review Boards from Louisiana State University and Tulane University Medical Center.

The HAPI Heart Study (n = 868) is a community-based study of cardiovascular risk carried out in the Amish community from Lancaster County, Pennsylvania [9]. Virtually all participants have descended from approximately 200 founders of the Old Order Amish community [10]. The study was approved by the appropriate Institutional Review Boards and monitored by an external Data Safety Monitoring Board.

DNA methylation measurements

GOLDN
Procedures for quantifying DNA methylation in GOLDN have been extensively described in previous publications [12,13]. Briefly, we restricted our analyses to CD4+ T-cells, isolated from peripheral blood frozen buffy coat samples, to reduce confounding by cell type. Upon isolating DNA using the DNeasy Kits (Qiagen, Venlo, Netherlands), we used the Infinium Human Methylation 450 Beadchip (Illumina, San Diego, CA) to measure genome-wide DNA methylation [12]. Subsequently, we estimated β scores, defined as the proportion of total signal from the methylation-specific probe or color channel, and detection p-values, defined as the probability that the total intensity for a given probe falls within the background signal intensity, using the GenomeStudio software provided by the manufacturer. For reasons of quality control, we did not analyze any β scores with an associated detection p-value greater than 0.01, samples with more than 1.5% missing data points across ~470,000 autosomal CpGs, or probes where 10% of samples or more failed to yield adequate intensity [12]. After filtering, we normalized the resulting β scores using the ComBat package for R software in nonparametric mode to account for batch effects [14]. Normalization for probes from the Infinium I and II chemistries was done separately, with subsequent adjustment of the Infinium II scores as previously described [12]. As the final quality control step, we identified and removed cross-reactive probes [12] and CpGs sites with SNPs on the probe as reported by Barfield et al. [15]. A total of 991 samples and 368,051 CpG sites remained in the analysis.

HAPI Heart Study
For the replication study, we extracted DNA from whole blood collected in acid citrate dextrose tubes (BD, Franklin Lakes, NJ) using the Gentra Puregene kit (Qiagen, Venlo, Netherlands). According to the manufacturer's protocol, we treated 1 μg of genomic DNA with bisulfite using the Epitect Fast 96 DNA bisulfite kit (Qiagen, Venlo, Netherlands). We obtained PyroMark CpG assays (Qiagen, Venlo, Netherlands) to quantify methylation on the top finding from GOLDN, located at CpG site cg00574958. To that end, we amplified 1 μl of bisulfite converted DNA using the PyroMark PCR kit (Qiagen, Venlo, Netherlands) with the supplied parameters. We determined PCR success by gel electrophoresis in 1.75% agarose gel. We performed pyrosequencing on 10 μl of PCR product using sequencing primer GTTGGTTTGGGGATTATTA and the GTATTYGTAG

TTGGTTTTTTGGATTGGTTTTG sequence to analyze. We successfully pyrosequenced a total of 726 samples. Of those, 543 samples had PCR reactions carried out in duplicate on two different machines, with the remaining 183 samples analyzed only on a single machine. The methylation measures utilized in the analysis correspond to samples with duplicate measures, using the average of the two methylation values. We additionally assessed the reproducibility of methylation measurements in the paired samples and removed observations for whom the coefficient of variation exceeded 20%, leaving 474 participants for the final analysis.

BHS

We extracted genomic DNA from 835 whole blood samples using the PureLink Pro 96 Genomic DNA Kit (Life-Technology, CA) following the manufacturer's instructions. Identically to GOLDN, we used the Infinium Human-Methylation450 BeadChip to quantify DNA methylation. All the DNA samples were processed at the Microarray Core Facility, University of Texas Southwestern Medical Center at Dallas, Texas. The image was acquired with HiScan scanner and analyzed with GenomeStudio software. The call rates for all samples across all CpG sites exceeded 99.8%. We used the latest version (1.2) of the Illumina Human Methylation 450k annotation in the current study to get the corresponding location information. Similarly to GOLDN, we converted DNA methylation levels at CpG sites into β scores (analogous to the proportion of DNA methylated), a continuous variable between 0 (unmethylated) and 1 (fully methylated). We normalized the data using the R package *watermelon* [16]. For correction of systematic technical biases in the 450K assay, we normalized β scores using the *dasen* function, in which type I and type II intensities and methylated and unmethylated intensities are quantile-normalized separately after backgrounds equalization of type I and type II probes. Based on bead count and detection p-values, we set the following thresholds for removal: 1) samples having 1% of CpG sites with a detection p-value greater than 0.05; 2) probes having 5% of samples with a detection p-value greater than 0.05; 3) probes with bead count less than 3 in 5% of samples.

Adiponectin measurements

As described in a prior GOLDN publication [17], we assayed plasma adiponectin using an ELISA kit from R&D Systems (Minneapolis, MN). We centrifuged all samples at $2000 \times g$ for 15 min at $4\,^{\circ}C$ within 20 min of collection, stored them frozen at $-70\,^{\circ}C$, and analyzed at the same time to eliminate batch effects. The reliability coefficient was estimated at 0.95 [17]. Both HAPI Heart Study and BHS measured adiponectin levels using a commercial radio-immunoassay kit (Linco Research, St Charles, MO). Adiponectin data were available on 474 HAPI Heart participants and 835 BHS participants with DNA methylation data that passed quality control exclusions as described above.

Statistical methods

The statistical methods for discovery and replication are summarized in Supplemental Table 1. Specifically, in all three populations we used linear mixed models to evaluate the association between plasma adiponectin (exposure) and DNA methylation β scores (outcome). To limit the undue influence of the skewed the adiponectin distribution on the regression coefficient estimates, we have ln-transformed it in all three populations.

Discovery

In GOLDN, we used linear mixed models to test for associations between methylation scores at each CpG site and ln-transformed plasma adiponectin adjusting for age, sex, study site, and the first 4 principal components generated to capture T-cell purity as fixed effects, and family as a random effect. We conducted sensitivity analyses additionally adjusting for body mass index (BMI), smoking, and alcohol to account for potential confounding. We constructed Manhattan plots to visualize the results.

Replication

We sought replication of all top hits (false discovery rate (FDR) <0.2) from GOLDN in the BHS cohort, which had extant methylation data available. Because the HAPI Heart cohort did not have array measurements of methylation and instead performed de novo pyrosequencing, we limited that replication effort to the top GOLDN CpG (FDR <0.05). In BHS, we fit linear mixed models adjusted for age, sex, and white blood cell differential cell counts (CD8T, CD4T, NK, B cells, monocytes, and granulocytes) as fixed effects and batch as a random effect. BHS analyses were stratified by race. The replication analysis in BHS used α = 0.05/number of CpGs with FDR <0.2 in GOLDN = 0.05/34 = 0.001. For the GOLDN hits that successfully replicated in at least one racial subgroup of BHS, we fit additional models including 1) BMI and 2) smoking and alcohol intake as sensitivity analyses.

In HAPI Heart, the models adjusted for age, sex, and batch as fixed effects as well as accounted for the family structure by including the relationship matrix as a random effect. We fit additional models with BMI and smoking as covariates to examine robustness of the finding. Because only one CpG locus was tested in HAPI Heart, this replication analysis used α = 0.05 as the significance threshold.

Although the discovery analysis did not include batch as a covariate because its effects were removed during the normalization phase using ComBat, we did account for batch in statistical models fit to the replication cohorts. Results from all three studies were meta-analyzed using Fisher's method [18], which makes no assumptions about individual effect sizes, as the estimates of effect were not directly comparable due to differences in cell types and modeling approaches.

Integration

In light of the evidence linking sequence variation to epigenetic patterns, we have attempted integrating signals

from prior genome-wide linkage and association studies of adiponectin in GOLDN with the epigenetic data. Specifically, we have performed look-up of methylation variants located in or near (+/−50 bp) the genes previously identified as implicated in adiponectin homeostasis in our study population: *ADIPOQ, CDH13, IL22RA1, IL28RA, PCSK5, SCUBE1*, and *TMEM18*, as well as intergenic variants on chromosomes 12 and 20 [7,17]. We also interrogated methylation in the following adiponectin-related genes identified in two or more populations in the GWAS catalog: *ARL15, CMIP, FER, GPR109A,* and *PEPD* (http://www.ebi.ac.uk/gwas/search?query=Adiponectin_levels). To conduct the lookup, we obtained regression coefficients and P-values for each CpG located in or near (+/−50 bp) the aforementioned candidate genes in the epigenome-wide study results from the discovery cohort (GOLDN).

Results

Table 1 summarizes the general characteristics of the study populations. Mean adiponectin concentrations were the highest among Amish participants and lowest among Black BHS participants; the inverse pattern was observed for BMI. On average, study participants were overweight but not obese.

We identified one locus in the first intron of *CPT1A* that was methylated in a direct correlation with plasma adiponectin (i.e. individuals with higher plasma adiponectin levels had higher methylation scores at this CpG site). In addition to the *CPT1A* locus, there were suggestive associations (FDR <0.20) for CpG sites in *FAM166B, SORL1, RNF145, UFM1,* and *ASAM* genes, among others. Table 2 shows the location, strength of association, and annotation for the top hits while Fig. 1 summarizes epigenome-wide results. Upon adjustment for BMI (Table 3), the association with the *CPT1A* locus diminished but remained statistically significant; however, the association estimates for other loci weakened substantially, yielding only two CpGs with FDR <0.20. In the models that adjusted for current smoking status or alcohol intake (Supplemental Table 2), the estimate of effect did not appreciably change compared with the baseline model.

Out of the 34 CpGs that were tested for replication in BHS, only 3 reached statistical significance in the White subset upon adjusting for multiple testing: cg00574958 in *CPT1A*, cg19750657 in *UFM1*, and cg26403843 in *RNF145* (Table 2), whereas none were significant in the smaller Black subsample. Adjustment for either BMI or smoking

attenuated the observed association with both loci in the BHS White participants, rendering them no longer statistically significant (cg00574958: P = 0.14 and 0.15, cg19750657: P = 0.01 and 0.02, cg26403843: P = 0.005 and 0.006 when adjusted for BMI and smoking plus alcohol, respectively).

Subsequently, the association with *CPT1A* locus was confirmed in the HAPI Heart study (Table 4). In the 474 participants of the HAPI Heart study, ln-transformed adiponectin levels were significantly and positively associated with cg00574958 methylation, similarly to GOLDN and BHS. The difference in the magnitude of the regression coefficients between HAPI Heart analyses and the other two cohorts could potentially be explained by different methylation measuring techniques (array vs. pyrosequencing). In HAPI Heart participants, the association diminished but remained significant upon adjustment for BMI (Table 4). Adjusting for smoking had a similar effect in HAPI Heart (Table 4). The association between methylation at the *CPT1A* locus and circulating adiponectin in the meta-analysis of all 2300 participants was highly significant: P = 4.4×10^{-16}.

Among genes harboring known sequence variants associated with circulating adiponectin, none contained epigenome-wide significant (FDR <0.20) methylation hits. The top locus among such candidate genes (regression coefficient (SE) = −0.003 (0.0008), P = 0.003) maps to *CDH13* on chromosome 16.

Discussion

The evidence in support of epigenetic regulation of complex traits such as plasma adiponectin is growing at a fast pace. However, prior to our study, investigations of epigenetic determinants of adipocytokines were limited to candidate gene analyses, usually involving the adiponectin gene itself (*ADIPOQ*) or its receptor (*ADIPOR1*), or other candidate genes such as *CDH13* [19–21]. The advent of high-resolution arrays enabled epigenome-wide profiling and discovery of novel loci such as the one we identified and successfully replicated in *CPT1A, UFM1*, and *RNF145*. It is highly likely that the observed association between all three loci and adiponectin levels are related to obesity, as evidenced by the results of our sensitivity analyses.

The differentially methylated locus identified and replicated in our study is located in the first intron of *CPT1A,* which encodes carnitine palmitoyltransferase 1A, the rate-limiting enzyme for mitochondrial fatty acid

Table 1 Demographic and clinical characteristics of the study populations.

	GOLDN (n = 991)	HAPI (n = 474)	BHS white (n = 592)	BHS black (n = 243)
Age, years[a]	49 ± 16	44 ± 14	43 ± 5	43 ± 5
Sex, n female (%)	518 (52)	221 (47)	318 (54)	157 (65)
Plasma adiponectin, μg/dL	8.3 ± 4.7	11.3 ± 5.9	8.9 ± 4.2	7.5 ± 4.0
Current smokers, n (%)	73 (7)	51 (11)	151 (26)	74 (30)
Body mass index, kg/m^2	28.3 ± 5.7	26.6 ± 4.4	28.6 ± 6.3	31.6 ± 8.3

[a] Values for continuous variables are shown as mean or median ± SE.

Table 2 Top CpG methylation sites associated[a] with plasma adiponectin in GOLDN (n = 991) (false discovery rate <0.2) and corresponding estimates of association from the replication analysis[b] in BHS (White n = 592, Black n = 243). Associations with CpGs that successfully replicated in at least one BHS racial group (P < 0.05/34 = 0.001) are in bold.

| Marker	Chromosome	Region	Gene	GOLDN		BHS white		BHS black	
				Regression Coefficient ± SE	P-value	Regression coefficient ± SE	P-value	Regression coefficient ± SE	P-value
cg00574958	**11**	**North shore**	***CPT1A***	**0.01 ± 0.001**	**3.4×10^{-13}**	**0.006 ± 0.001**	**0.0005**	**0.0006 ± 0.003**	**0.18**
cg21212076	9	–	FAM166B	−0.02 ± 0.003	7.8×10^{-7}	−0.002 ± 0.004	0.61	0.001 ± 0.005	0.85
cg22964469	11	–	SORL1	0.007 ± 0.001	8.8×10^{-7}	0.0009 ± 0.002	0.56	0.004 ± 0.002	0.01
cg14266156	17	–	Intergenic	0.007 ± 0.001	1.6×10^{-6}	−0.0004 ± 0.002	0.78	−0.002 ± 0.003	0.56
cg19828789	5	–	Intergenic	−0.008 ± 0.002	2.8×10^{-6}	0.0003 ± 0.002	0.84	−0.0008 ± 0.002	0.66
cg05358404	20	Island	RTEL1	0.02 ± 0.003	2.9×10^{-6}	0.006 ± 0.004	0.21	−0.0002 ± 0.005	0.96
cg05931265	11	South shore	UBASH3B	0.006 ± 0.001	3.0×10^{-6}	−0.0004 ± 0.002	0.86	−0.006 ± 0.003	0.05
cg19393762	19	–	TNNT1	−0.009 ± 0.002	3.6×10^{-6}	−0.002 ± 0.002	0.34	−0.0002 ± 0.003	0.95
cg07895124	16	–	GPR114	−0.008 ± 0.002	4.1×10^{-6}	−0.0004 ± 0.002	0.81	−0.0003 ± 0.003	0.89
cg25649039	17	–	PRKCA	−0.01 ± 0.002	4.6×10^{-6}	−0.002 ± 0.002	0.47	−0.004 ± 0.003	0.24
cg24787076	5	North shore	RPL37	0.004 ± 0.0009	4.7×10^{-6}	0.002 ± 0.001	0.09	0.003 ± 0.002	0.09
cg19750657	**13**	**–**	***UFM1***	**−0.008 ± 0.002**	**5.2×10^{-6}**	**−0.01 ± 0.003**	**0.0002**	**0.003 ± 0.006**	**0.66**
cg05879734	2	–	Intergenic	0.003 ± 0.0007	6.1×10^{-6}	0.002 ± 0.001	0.12	0.005 ± 0.002	0.02
cg26797723	3	Island	WNT5A	−0.004 ± 0.001	6.3×10^{-6}	0.0005 ± 0.0006	0.46	−0.003 ± 0.001	0.008
cg18810022	2	–	Intergenic	0.008 ± 0.002	5.4×10^{-6}	0.0008 ± 0.002	0.60	−0.0002 ± 0.002	0.93
cg26985149	17	Island	Intergenic	0.005 ± 0.001	7.4×10^{-6}	−0.002 ± 0.002	0.34	0.0002 ± 0.003	0.94
cg06710295	8	Island	MTSS1	0.002 ± 0.0005	7.8×10^{-6}	0.0001 ± 0.001	0.89	0.0004 ± 0.001	0.74
cg16597079	6	–	TRERF1	0.006 ± 0.001	7.9×10^{-6}	−0.0001 ± 0.001	0.92	0.002 ± 0.002	0.23
cg11552078	19	South shore	TIMM50	0.005 ± 0.001	7.9×10^{-6}	0.001 ± 0.002	0.62	−0.006 ± 0.003	0.03
cg18758976	6	–	SYNJ2	0.007 ± 0.002	7.9×10^{-6}	−0.001 ± 0.001	0.36	−0.0003 ± 0.002	0.88
cg18244544	19	North shore	ZNF224	0.002 ± 0.0004	8.9×10^{-6}	0.0003 ± 0.001	0.70	0.0009 ± 0.001	0.36
cg26894079	11	–	ASAM	0.01 ± 0.002	9.0×10^{-6}	0.006 ± 0.003	0.02	0.006 ± 0.004	0.14
cg18098839	3	–	GOLIM4	0.004 ± 0.001	9.7×10^{-6}	0.005 ± 0.002	0.005	0.002 ± 0.003	0.44
cg00325866	15	North shore	C2CD4A	0.001 ± 0.0002	9.9×10^{-6}	−0.0004 ± 0.0005	0.45	−0.0006 ± 0.0007	0.37
cg15183258	15	–	MGA	−0.02 ± 0.004	1.0×10^{-5}	−0.001 ± 0.003	0.62	0.001 ± 0.004	0.77
cg14652230	3	North shore	NICN1	0.002 ± 0.0005	1.1×10^{-5}	−0.0006 ± 0.0008	0.46	0.001 ± 0.001	0.41
cg09251317	14	–	Intergenic	0.008 ± 0.002	1.4×10^{-5}	−0.0004 ± 0.002	0.80	0.007 ± 0.003	0.01
cg26403843	**5**	**North shelf**	***RNF145***	**−0.008 ± 0.002**	**1.5×10^{-5}**	**−0.02 ± 0.004**	**0.0004**	**−0.01 ± 0.006**	**0.04**
cg08775774	12	–	CCDC62	0.002 ± 0.0004	1.5×10^{-5}	−0.0005 ± 0.0006	0.47	−0.0002 ± 0.0008	0.83
cg17593721	5	South shore	HSD17B4	0.003 ± 0.0007	1.5×10^{-5}	0.0004 ± 0.001	0.73	−0.003 ± 0.002	0.09
cg23923820	1	South shore	PKLR	−0.01 ± 0.002	1.5×10^{-5}	−0.0004 ± 0.002	0.85	−0.003 ± 0.004	0.41
cg05466767	16	North shelf	FTSJD1	0.005 ± 0.001	1.6×10^{-5}	−0.0009 ± 0.0007	0.18	−0.002 ± 0.001	0.58
cg19595760	1	South shelf	MAN1C1	0.009 ± 0.002	1.8×10^{-5}	0.001 ± 0.002	0.63	−0.0008 ± 0.003	0.82
cg14584616	7	North shelf	HEATR2	−0.008 ± 0.002	1.8×10^{-5}	0.0001 ± 0.001	0.91	−0.001 ± 0.002	0.58

[a] Association estimates in GOLDN were obtained from linear mixed models with ln-transformed adiponectin as a predictor and methylation β score as the outcome, adjusted for age, sex, study site, T-cell purity principal components (fixed effects) and family relatedness (random effect).
[b] Association estimates in BHS were obtained from linear mixed models with ln-transformed adiponectin as a predictor and methylation β score as the outcome, adjusted for age, sex, white blood cell counts (fixed effects) and batch (random effect).

oxidation. Prior studies—including several from GOLDN—have linked methylation of *CPT1A* to 1) reduced expression of *CPT1A* and 2) obesity indices, plasma triglycerides, very low-density lipoprotein cholesterol, and metabolic syndrome, highlighting the biological relevance of the observed association [6,13,22]. Specifically, the study linking *CPT1A* methylation to circulating lipids also provided functional annotation data, illustrating the regulatory potential of the top CpG site: in addition to several transcription factor binding sites, our top locus is also adjacent (upstream) to an active promoter region, and overlaps an H3K27Ac histone mark [6]. Interestingly, prior analyses of the GOLDN data [6] do not report significant sequence variant effects (methylation quantitative trait loci) in that region. Other groups have found hepatic expression of *CPT1A* to be correlated with the amount of liver fat, and the correlation decreased when adjusted for BMI [23]. In the GOLDN and HAPI studies, adjustment for

BMI similarly, albeit incompletely, attenuated the estimate of *CPT1A* effect. In contrast, the association between *CPT1A* and plasma adiponectin was not observed at all among Black participants from BHS, and was eliminated completely upon adjustment for BMI among White participants. Considering the small size of the Black sample, this may represent limitations of statistical power rather than true effect modification by race; in fact, *CPT1A* methylation was confirmed to be associated with BMI in a much larger African American sample from the Artherosclerosis Risk in Communities study [13].

Previously published evidence shows that the relationship between epigenomic variation, obesity, and adiponectin is likely to be complex and multidirectional. For example, in a recent animal study, obesity has been shown to mediate insulin resistance by inducing hypermethylation of the adiponectin gene [4], while in a small-scale human trial both obesity and insulin resistance
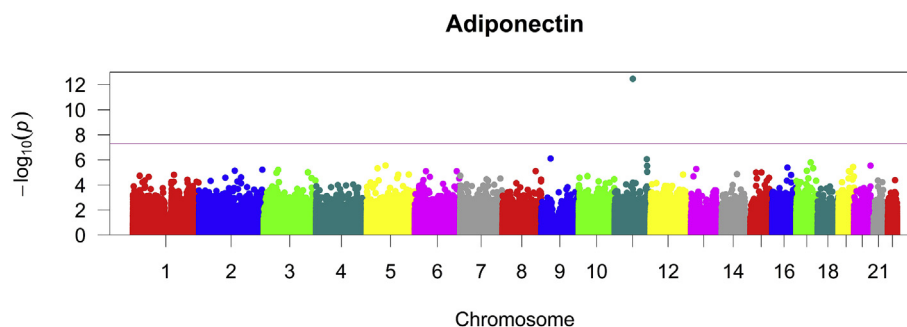
Adiponectin

Figure 1 Epigenome-wide associations of CpG methylation with plasma adiponectin in GOLDN (n = 991). Manhattan plot of epigenome-wide results of testing for association between methylation at 368,051 CpG sites and plasma adiponectin. The X-axis displays the chromosome on which the site is located, the Y-axes display −log10(P-value). The red horizontal line indicates the threshold for epigenome-wide statistical significance after a Bonferroni correction (P-value < 0.05/368,051 = 1.4×10^{-7}). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3 Top CpG methylation sites associated[a] with plasma adiponectin (false discovery rate <0.2) in the discovery population (n = 991), adjusted for body mass index.

Marker	Chromosome	Region	Gene	Regression Coefficient ± SE	P-value
cg00574958	11	North shore	*CPT1A*	0.008 ± 0.001	1.9×10^{-8}
cg24787076	5	North shore	*RPL37*	0.005 ± 0.0009	2.3×10^{-7}

[a] Association estimates were obtained from linear mixed models with ln-transformed adiponectin as a predictor and methylation β score as the outcome, adjusted for age, sex, BMI, study site, T-cell purity principal components (fixed effects) and family relatedness (random effect).

actually diminished methylation of both leptin and adiponectin promoters [24]. It is noteworthy that methylation of *ADIPOQ* or any other known adiponectin loci was associated with neither obesity [13] nor insulin resistance [25] nor adiponectin in GOLDN, suggesting alternative pathways of epigenetic regulation.

In addition to the *CPT1A* locus, we have identified and replicated associations with CpG sites located in *UFM1* and *RNF145*. The cg19750657 locus is located in the 3′ UTR region of *UFM1* (ubiquitin-fold modifier 1), which encodes a highly conserved posttranslational modifier involved in endoplasmic reticulum functions and other cellular processes [26]. Specifically, it is highly expressed in pancreatic islets and upregulated in type 2 diabetes, representing a protective response to endoplasmic reticulum stress [27]. The cg19750657 site in particular has been shown to be hypermethylated in adipose tissue in the setting of type 2

diabetes [28], consistent with our observed inverse association with plasma adiponectin. Although its location in the 3′ UTR region (Fig. 2) makes it an unlikely candidate for influencing expression, prior studies show that methylation of the untranslated regions may exert other forms of epigenetic control, notably transcription elongation [29]. Related to the ubiquitin pathway represented by our *UFM1* finding is the ring zinger protein encoded by *RNF145*, which includes the other replicated methylation locus. Namely, it regulates an ubiquitin-cycling node in the oxidative burst response and is involved in endopasmic reticulum-associated degradation [30]. Notably, methylation of cg26403843 in *RNF145* has been previously linked to BMI and waist circumference in several diverse populations [31,32]. Additionally, the cg26403843 site colocalizes with an H3K27Ac histone mark in immune cells from the ENCODE data, suggesting an active regulatory

Table 4 Associations between the methylation of the cg00574958 locus in *CPT1A* in the discovery and replication cohorts.

Cohort	N	Basic model[a]		Basic model[a] + BMI		Basic model[a] + smoking + alcohol[b]	
		Regression Coefficient ± SE	P-value	Regression Coefficient ± SE	P-value	Regression Coefficient ± SE	P-value
GOLDN	991	0.01 ± 0.001	3.4×10^{-13}	0.008 ± 0.001	1.9×10^{-8}	0.01 ± 0.001	7.4×10^{-14}
HAPI Heart	474	0.88 ± 0.26	0.0009	0.61 ± 0.28	0.03	0.60 ± 0.27	0.03
BHS (White)	592	0.006 ± 0.002	0.0005	0.002 ± 0.002	0.14	0.002 ± 0.002	0.15
BHS (Black)	243	0.003 ± 0.002	0.18	0.0006 ± 0.003	0.79	0.0008 ± 0.003	0.74

[a] Basic model refers to a linear mixed model with ln-transformed adiponectin as a predictor and methylation β score as the outcome, adjusted for age, sex, technical covariates (in GOLDN: study site and T-cell purity principal components; in HAPI Heart: batch; in BHS: white blood cell counts and batch), and family relatedness (only in GOLDN and HAPI Heart).
[b] Alcohol intake data was not available in HAPI Heart but is likely to be minimal due to religious restrictions; therefore, the association estimates for HAPI Heart in this column are derived from basic model + smoking.
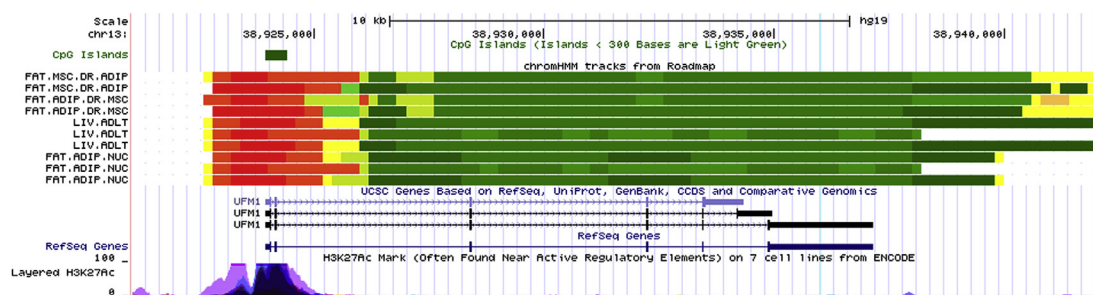
Figure 2 ENCODE/Roadmap Epigenomics Project annotation of the genomic region containing the *UFM1* methylation locus on chromosome 13, generated using data from the ENCODE/Roadmap Epigenomics Project. Functional features are listed on the Y-axis, while the X-axis represents the genomic position (hg19, Human Genome Build 37). Notable features include (from top to bottom): CpG islands, chromatin state across tissues physiologically relevant to adiponectin regulation (adipose and hepatic), known genes, layered H3K27Ac marks commonly found near active regulatory elements. The location of the CpG is marked by the faint blue vertical line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
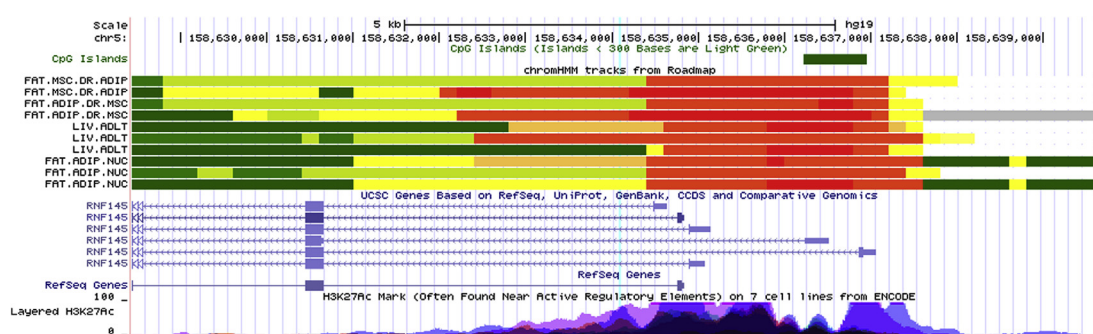
Figure 3 ENCODE/Roadmap Epigenomics Project annotation of the genomic region containing the *RNF145* methylation locus on chromosome 5, generated using data from the ENCODE/Roadmap Epigenomics Project. Functional features are listed on the Y-axis, while the X-axis represents the genomic position (hg19, Human Genome Build 37). Notable features include (from top to bottom): CpG islands, chromatin state across tissues physiologically relevant to adiponectin regulation (adipose and hepatic), known genes, layered H3K27Ac marks commonly found near active regulatory elements. The location of the CpG is marked by the faint blue vertical line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

element (Fig. 3). However, despite successful replication across cohorts, these findings need to be interpreted with caution because the strength of the association was attenuated upon adjustment for either BMI or smoking + alcohol, indicating potential influence of known epigenetic confounders.

Like most population-based studies of epigenetic patterns published to date, our study is cross-sectional and restricted to blood samples. We argue that the use of CD4+ T cells (discovery cohort) as well as whole blood samples (replication) represent a strength rather than a weakness of our study, as these are easily accessible tissues with high translational potential. Importantly, a previous study has reported high correlation of DNA methylation levels at biologically relevant (with regard to leptin and adiponectin regulation) CpGs between blood and adipose tissue (both visceral and subcutaneous), corroborating the validity of our strategy [33]. To account for the dynamic nature of epigenetic processes, our findings would best be followed up by investigating links between gene methylation/expression and adipocytokines in a longitudinal setting to control for reverse causation. Additionally,

future studies would benefit from attempting validation of our preliminary findings of other adiponectin-related methylation loci, furthering current understanding of the genetic architecture of circulating adiponectin.

In summary, we have conducted the first epigenome-wide study of circulating adiponectin levels. We have shown that methylation of a CpG site in *CPT1A* is associated with circulating adiponectin levels, likely in an obesity-dependent manner, in three population-based adult cohorts of European descent. Our findings enhance current understanding of genetic architecture of plasma adiponectin levels and lay the groundwork for testing *CPT1A* methylation as a novel pleiotropic marker of chronic disease risk.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2016.11.004.

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