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DNA methylation markers of insulin resistance surrogate measures in the Atherosclerosis Risk in Communities (ARIC) study

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ABSTRACT

Insulin resistance (IR) is a risk factor for cardiovascular diseases and type 2 diabetes. Associations between DNA methylation (DNAm) and IR have been less studied in African ancestry (AA) populations than those of European ancestry (EA). We aimed to identify associations between whole blood DNAm and IR in up to 1,811 AA and 964 EA participants from the Atherosclerosis Risk in Communities (ARIC) study. We quantified IR using three surrogate measures: the homeostasis model assessment of insulin resistance (HOMA-IR), the triglyceride-glucose index (TyG), and the triglyceride glucose-body mass index (TyG-BMI). We used ancestry-stratified linear regression models to conduct epigenome-wide association studies of IR, adjusting for batch effects and relevant covariates. Among 484,436 tested CpG sites, 39 were significantly associated with IR, of which 31% (10 in AA and two in EA) were associated with TyG-BMI and not previously reported for IR or related traits. These include a positive association at cg18335991-*SEMA7A* in AA. *SEMA7A* inhibits adipogenesis of preadipocytes and lipogenesis of mature adipocytes. DNAm levels at cg18335991 have been reported to be negatively associated with *SEMA7A* expression in blood. After additionally adjusting for smoking and drinking status, 15 of the 39 significant CpG sites remained significant or suggestive. Our study identified novel IR-associated CpG sites, contributing to a broader understanding of the epigenetic mechanisms underlying IR in diverse populations.

KEY POINTS HIGHLIGHTS

- Associations between DNA methylation (DNAm) and insulin resistance (IR) have been primarily studied in individuals of European ancestry. The epigenetic regulation of IR, quantified using different surrogate measures, has not been extensively reported in diverse populations.
- This study aimed to identify associations between DNAm and IR in African and European ancestries and assessed how these associations differ by IR surrogate measures.
- Among 39 significant IR-associated CpG sites, 12 CpG sites, associated with the triglyceride glucose-body mass index, were potentially novel, and most were detected in African ancestry participants.
- This study improves our understanding of the epigenetic mechanisms underlying IR in diverse populations.

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Introduction

Insulin resistance (IR) is defined as an impaired response of the body to insulin action, increasing glucose concentrations in blood and pancreatic β-cell insulin production. IR is a risk factor for cardiovascular diseases (CVD) [1], type 2 diabetes [2] and metabolic syndrome (MetS) [3]. Differences in the prevalence/incidence of diabetes between African ancestry (AA) and European ancestry (EA) population groups have been examined in the National Health and Nutrition Examination Survey (NHANES) [4] and the Atherosclerosis Risk in Communities (ARIC) study [5]. Population group differences in IR have also been studied between AA and EA populations [6,7]. Both genetic and epigenetic

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factors have been previously reported to be associated with IR [8,9]. Environmental and social exposures can cause epigenetic changes and impact health outcomes and risk of disease. Therefore, it is important to evaluate the association of CpG sites with IR in different population groups. Previous epigenome-wide association studies (EWAS) of IR have been conducted primarily in EA populations, and only a few studies were conducted in AA populations (Table S1) [10–16]. The EWAS conducted for IR in AA populations may have been underpowered due to small sample sizes. For instance, Chilunga et al. identified three differentially methylated positions associated with homeostasis model assessment of insulin resistance (HOMA-IR) in blood samples of AA men ($N = 136$) from the Howard University Family Study [10]. Sharma et al. identified 155 CpG sites associated with Matsuda index in adipose tissue of AA participants ($N = 230$) from the African American Genetics of Metabolism and Expression (AAGMEx) cohort [16].

Insulin sensitivity can be measured using hyperinsulinemic-euglycemic clamp technique [17], although doing so in a clinical setting is difficult because it is invasive, expensive, and labour intensive. Hence, several IR surrogates have been used to quantify insulin sensitivity. Most EWAS of IR were conducted using HOMA-IR derived from fasting insulin and fasting glucose (Table S1) [10–14,18]. The triglyceride-glucose (TyG) index has been recently proposed as an easily accessible and reliable alternative IR surrogate, based on fasting glucose and fasting triglycerides [19]. The biological basis of using TyG as an IR surrogate is that as insulin regulates lipolysis in adipocytes, IR affects lipid metabolism and can lead to dyslipidemia and high triglyceride level [1]. A previous study reported a significant correlation (Pearson correlation $r = -0.681$, $p < 0.005$) between TyG and hyperinsulinemic-euglycemic clamp [20]. TyG has been shown to be associated with CVD, type 2 diabetes, and MetS [21–23]. Obesity can lead to adipose cell dysfunction and IR [24]. Because the body mass index (BMI) is a simple measurement of obesity and is highly associated with metabolic risk, a combination of TyG and BMI, triglyceride glucose-body mass index (TyG-BMI), has been shown to perform better at identifying earlier IR [25]. Some studies have suggested that TyG-BMI is a more powerful marker for IR relative to other commonly used IR surrogates [25].

In this study, we conducted an EWAS of IR in AA and EA participants from the ARIC study using three different surrogate measures of IR (HOMA-IR, TyG, and TyG-BMI) to identify associations between DNA methylation (DNAm) and IR in two population groups and observe how these associations differ by IR measures.

Materials and methods

Study population

The ARIC study is a population-based prospective cohort study of cardiovascular disease sponsored by the National Heart, Lung, and Blood Institute (NHLBI). ARIC included 15,792 individuals, predominantly of European ancestry ($N = 11{,}478$) and African ancestry ($N = 4{,}266$), aged 45–64 years at baseline (1987–89), chosen by probability sampling from four US communities. Participants from three communities, including Minneapolis, MN; Washington County, MD; and Forsyth County, NC, were recruited from the general population; while participants from Jackson, MS, were all of African ancestry [26]. The first four cohort study examinations were conducted every 3 years, in 1987–1989 (visit 1), 1990–1992 (visit 2), 1993–1995 (visit 3), and 1996–1998 (visit 4). The fifth examination was carried out in 2011–2013 (visit 5), followed by a sixth exam in 2016–2017, a seventh exam in 2018–2019, an eighth exam in 2020, a ninth exam in 2021–2022, a tenth exam in 2023, and the most recent examination (visit 11) in 2024–2025. In this study, we focused on visits 1, 2, and 3, based on the availability of DNA methylation and IR surrogate measures. After excluding ARIC participants who self-identified as Asian ($N = 34$), American Indian or Alaskan Indian ($N = 14$), participants in our study represent two major self-reported population groups (African American and European American).

DNA methylation

DNAm data measured in peripheral blood were obtained after bisulfite conversion of genomic

DNA with the EZ-96 DNA Methylation Kit (Deep Well Format) (Zymo Research; Irvine, CA, USA) using the Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA) in accordance with the manufacturer's instructions. Detailed information on DNAm data and quality control procedures [27–30] is provided in the Supplementary Material. DNAm data were available for AA and EA participants and were collected at either visit 2 ($N_{AA} = 2{,}079$ and $N_{EA} = 761$) or visit 3 ($N_{AA} = 340$ and $N_{EA} = 282$). A total of 484,436 CpG sites (483,525 in AA and 482,815 in EA) were analysed in the EWAS.

Genotyping and imputation

Genotype data for ARIC was completed using the Affymetrix Array 6.0. Quality metrics used to filter samples and variants before imputation are described hereafter. Sample QC included filtering out sex mismatch, first-degree relatives of an included individual, genetic outliers based on allele sharing and principal component analyses, and participants with low call-rate (<95%). Variant QC included filtering out monomorphic SNPs, low call rate <95%, minor allele frequency (MAF) <0.01, and deviation from Hardy-Weinberg equilibrium (HWE, P-value <1E–05). Imputation of the genotype data using the Trans-Omics for Precision Medicine (TOPMed) R2 reference panel was performed using the TOPMed Imputation server [31–33].

Surrogate measures of IR

For participants who fasted for 8 h or more, fasting glucose and triglycerides were measured at visits 1, 2, and 3, and fasting insulin was collected only at visit 1. Formulas used to derive HOMA-IR, TyG, and TyG-BMI were as follows: HOMA-IR = Fasting glucose [mg/dL] × Fasting insulin [μU/mL]/405 [34] at visit 1 (Figure S1); TyG = ln(Fasting triglycerides [mg/dL] × Fasting glucose [mg/dL]/2) [19] and TyG-BMI = TyG × BMI [kg/m^2] [25] at either visit 2 or visit 3, matching the visit of DNAm measurement (Figure S1).

Statistical analysis

Covariates and EWAS model

We assessed the association of DNAm with IR surrogate measures in AA and EA separately using linear regression models. In each ancestry-stratified analysis, we regressed DNAm beta values on each of the three different IR measures. We adjusted the analyses for batch effects using the first 25 principal components calculated for methylation control probes [35], self-reported sex, the estimated white blood cell counts (WBC), study center, visit at DNAm measurement, age at DNAm measurement, and BMI at IR measurement for HOMA-IR and TyG analyses (Figure S1). We matched BMI measurement visit to IR measurement visit. Additional details on the TyG-BMI EWAS model and rationale for not adjusting for BMI are provided in the Supplementary Material.

Because the distribution of HOMA-IR and TyG-BMI were right skewed, we log-transformed these variables. We used three linear regression models for each measure of IR stratified by ancestry:

HOMA-IR: DNAm ~ ln(HOMA-IR) + Age + BMI + Sex + Center + Visit + WBC + $PC_{ctrl}1$–25

TyG: DNAm ~ TyG + Age + BMI + Sex + Center + Visit + WBC + $PC_{ctrl}1$–25

TyG-BMI: DNAm ~ ln(TyG × BMI) + Age + Sex + Center + Visit + WBC + $PC_{ctrl}1$–25

We excluded participants who were not fasting for 8 h or more at the visit of the IR measurement and with diabetes at IR measurement. Diabetes was defined as non-fasting glucose level ≥200 mg/dL, fasting glucose level ≥126 mg/dL, self-report of a physician diagnosis of diabetes, or self-report of medication use in the past 2 weeks for diabetes or high blood sugar. After removing participants with diabetes, no fasting status at the IR measurement, or missing covariates, the number of remaining participants for each EWAS model was $N = 1{,}811$ for HOMA-IR analysis in AA, $N = 964$ for HOMA-IR analysis in EA, $N = 1{,}577$ for TyG and TyG-BMI analyses in AA and $N = 971$ for TyG and TyG-BMI analyses in EA.

After controlling for test statistic inflation and bias in EWAS using the BACON method [36],

significant associations were identified at the 0.05 level after a Bonferroni correction for the number of CpG sites tested ($N = 483,525$ in AA, $N = 482,815$ in EA, total number of CpG sites including non-variable or extreme beta values) and three IR measures (P-value <3.45E–08 for EA and AA). The minimum proportion of variance explained by each of the six EWAS models to achieve 80% power at P-value <3.45E–08 ranged between 0.046 and 0.088. We defined suggestive evidence of association using a threshold of 0.05 level after a Bonferroni correction for the number of CpG sites tested, without correction for the three surrogate measures of IR (P-value <1.0E–07 for EA and AA). We did not include CpG sites that were non-variable or exhibited extreme beta values in downstream analyses.

Published EWAS of IR or IR-related traits

First, we performed a review of the literature for EWAS of IR. For each published EWAS of IR or individual traits used in IR surrogate calculations, Table S1 summarizes the characteristics of the study population and study design, including a measure of IR and population ancestry. We also used the EWAS Catalog [37] and EWAS Atlas [38] to determine if the CpG sites significantly or suggestively associated with IR surrogates in this study were reported by previous IR or IR-related EWAS. IR traits that were considered included TyG, HOMA-IR, and variables used in IR surrogate derivation formula (glucose, insulin, and triglycerides). We additionally considered the EWAS results reported for IR-related traits including type 2 diabetes, BMI, lipids, blood pressure, and obesity [1,2].

Validation of significant associations across ancestries and IR phenotypes

We evaluated whether significant associations in one ancestry were observed in the other ancestry with consistent direction of effects at nominal significance (P-value < 0.05) for each IR measure. Similarly, we evaluated whether CpG sites significantly associated with one IR measure were associated with the other IR measures with consistent direction of effects at nominal significance in each ancestry group.

Sensitivity analyses

The association between DNAm and IR can be driven by CVD risk factors such as smoking and drinking status or can differ by sex. Therefore, we conducted two sensitivity analyses. First, given the significant correlations observed between smoking and drinking status and IR surrogate measures, we performed additional adjustment for both smoking and drinking status, each categorized as current, former, or never, at the time of DNAm measurement to measure their potential confounding effect on the association between DNAm and IR. We also reviewed whether CpG sites associated with IR surrogate measures in ARIC were previously reported by EWAS of smoking and alcohol consumption using the EWAS Catalog [37] and EWAS Atlas [38]. Second, to explore whether the effect of IR on DNAm varied by sex, we incorporated an interaction term between IR and sex into the EWAS models for the CpG sites significantly associated with IR.

Cis-mQTL, cis-eQTM, and cis-eQTL annotations

We assessed cis-acting DNAm quantitative trait loci (mQTLs) for the CpG sites significantly associated with IR in ARIC but not previously reported for IR or IR-related traits to examine if the target CpG sites have significant association with genetic variants near to or proximal to the CpG site. In the ARIC mQTL analyses, we considered genetic variants within a ± 500 kb window from each CpG site that had Rsq ≥ 0.8 and MAF ≥ 0.01 in the TOPMed-imputed ARIC genotype data. We first regressed DNAm beta values on age at DNAm measurement, WBC, sex, center, visit, $PC_{ctrl}1$–25, and genetic PCs 1–10. We then took the residuals from this model and regressed them on imputed dosages of variants, age, sex, center, and genetic PCs 1–10 to assess cis-mQTL associations. After excluding participants who had missing genotype data, DNAm data, or covariates, 1,486 AA and 865 EA participants were retained, and we tested 66,339 CpG-SNP pairs in AA and 40,139 CpG-SNP pairs in EA for the 12 potentially novel CpG sites. We used a Bonferroni correction for the number of CpG-

variant pairs at the 0.05 level to define the significance of mQTLs.

We also searched publicly available mQTL databases of blood samples including mQTL Database [39], BIOS QTL browser [40] and GoDMC mQTL [41], and a mQTL database of brain samples, Brain xQTLServe [42], where all four mQTL databases were from European populations. mQTLs associations can differ by ancestry, life (developmental) stage and tissue type. In the mQTL Database [39], we had access to mQTLs from whole blood samples of a middle-aged European population and in the BIOS QTL browser [40] and GoDMC mQTL [41], mQTLs were from whole blood samples of EA.

We also explored cis-expression quantitative trait methylation (cis-eQTMs) using the BIOS QTL browser to detect CpG-transcript pairs to find association between methylation and gene expression of genes near to or proximal to the significant CpG sites that have not been previously reported for IR or IR-related traits.

Finally, we used the GTEx Portal and the eQTLGen Consortium database [43,44] to find cis-eQTLs in blood that overlap with mQTLs.

Previous GWAS associations

At potentially novel CpG sites that were significantly associated with IR measures, and not previously reported by IR or IR-related traits EWAS, we used the GWAS Catalog [45] to find IR or IR-related GWAS associations for genes that were annotated to CpG sites.

Ethics approval and consent to participate

The ARIC study protocol was approved by the institutional review board of each participating institution. Written informed consent, including consent for genetic studies, was obtained from the ARIC participants.

Results

Demographic characteristics of the study sample

The demographic characteristics of ARIC participants in each EWAS model are presented in Table 1. Slightly more participants were included in the HOMA-IR analysis ($N = 2{,}775$ including 964 EA and 1,811 AA participants) than in the TyG or TyG-BMI analyses ($N = 2{,}494$ including 917 EA and 1,577 AA participants). The number of AA participants with DNAm data was larger than EA participants for all three IR surrogate measures analyses. In the three IR models, around 38–39% and 42% were males in AA and EA, respectively. The mean age at DNAm measurement in the three IR models was around 56–58 years (SD = 6) and 60 years (SD = 5) in AA and EA, respectively. Detailed comparisons of the characteristics of ARIC participants included in each EWAS of IR model and correlations among IR surrogates and the subcomponents are summarized in the Supplementary Material and Table S2.

Significant CpG associations with IR

Manhattan plots for EWAS of IR are presented in Figure 1 and Figure S2. The adjusted genomic inflation factor λ was close to 1 in all EWAS models, λ: [0.89,1.07], indicating no evidence of inflation (λ < 1.1). EWAS of HOMA-IR, TyG, and TyG-BMI across two ancestries identified 39 CpG sites significantly associated (P-value <3.45E–08) with at least one of the three IR measures (Table S3). The scatter plots of DNAm beta values vs. IR measures of the top 5 significant associations are presented in Figure S3. The TyG-BMI analysis detected the largest number of significant CpG sites (HOMA-IR, $N = 2$; TyG, $N = 8$; TyG-BMI, $N = 34$). We identified additional 10 CpG sites suggestively associated with IR at P-value <1.0E–07 (HOMA-IR, $N = 1$; TyG, $N = 2$; and TyG-BMI, $N = 7$). None of the 49 CpG sites detected suggestively associated with IR were non-variable or exhibited extreme beta values.

Potentially novel CpG sites associated with IR

As shown in Table 2, out of a total of 39 significant IR-associated CpG sites, 31% of them ($N = 12$) were potential novel CpG sites not previously reported by IR or IR-related traits EWAS regardless of the ancestry of the study population of the published EWAS studies. All of them were detected associated with TyG-BMI (10 from AA EWAS and two from EA EWAS). We assessed if the significant associations were detected only in one ancestry group (either AA or EA) because of the potential low power in the analysis

Table 1. Characteristics of the final set of ARIC participants included in each EWAS of IR model after excluding participants with diabetes.

HOMA-IR			TyG, TyG-BMI		
	African ancestry (AA, $N = 1811$)	European ancestry (EA, $N = 964$)		African ancestry (AA, $N = 1577$)	European ancestry (EA, $N = 917$)
Sex, N (%)			Sex, N (%)		
Male	700 (38.7)	408 (42.3)	Male	602 (38.2)	382 (41.7)
Female	1,111 (61.3)	556 (57.7)	Female	975 (61.8)	535 (58.3)
Age at visit 1 (years), mean (SD)	53.2 (5.7)	56.2 (5.1)	Age at visit 2 or 3 (years), mean (SD)	56.4 (5.8)	59.8 (5.4)
Age difference between visit 1 and 2 or 3 (years)*, mean (SD)	3.3 (1.2)	3.6 (1.4)	BMI at visit 2 or 3 [kg/m^2], mean (SD)	29.3 (6.0)	25.8 (4.2)
BMI at visit 1 [kg/m^2], mean (SD)	29.2 (5.9)	25.7 (4.0)	TyG, visit 2 or 3, mean (SD)	8.5 (0.5)	8.7 (0.5)
HOMA-IR, visit 1, mean (SD)	3.4 (2.8)	2.4 (1.9)	TyG-BMI, visit 2 or 3, mean (SD)	249.5 (54.6)	225.0 (42.2)
Smoking status*, visit 2 or 3, N (%)			Smoking status*, visit 2 or 3, N (%)		
Current	485 (26.8)	181 (18.8)	Current	427 (27.1)	173 (18.9)
Former	551 (30.4)	376 (39.0)	Former	483 (30.6)	355 (38.7)
Never	761 (42.0)	406 (42.1)	Never	666 (42.2)	389 (42.4)
Missing	14 (0.8)	1 (0.1)	Missing	1 (0.1)	0
Drinking status*, visit 2 or 3, N (%)			Drinking status*, visit 2 or 3, N (%)		
Current	647 (35.7)	535 (55.5)	Current	575 (36.5)	512 (55.8)
Former	551 (30.4)	135 (14.0)	Former	466 (29.5)	129 (14.1)
Never	599 (33.1)	293 (30.4)	Never	534 (33.9)	276 (30.1)
Missing	14 (0.8)	1 (0.1)	Missing	2 (0.1)	0

The ARIC participants have full consent to data sharing and DNAm data for analysis after excluding participants with diabetes or missing values. For descriptive statistics, mean (SD) was used for continuous variables and count N (%) was used for categorical variables. Prevalent diabetes was assessed using a derived variable using a blood glucose level (non-fasting glucose level ≥200 mg/dL, fasting glucose level ≥126 mg/dL), self-report of a physician diagnosis of diabetes, self-report of medication use in the past 2 weeks for diabetes or high blood sugar. HOMA-IR (homeostasis model assessment of IR) = Fasting glucose [mg/dL] × Fasting insulin: [μU/mL]/405. TyG (Triglyceride-glucose index) = ln(Fasting triglycerides [mg/dL] × Fasting glucose [mg/dL]/2). *Covariates for sensitivity analysis. Correlations between HOMA-IR, TyG, and TyG-BMI at visit 1 range from [0.37–0.57] in AA and [0.46–0.60] in EA. Correlations between TyG and TyG-BMI at DNAm measurement (visit 2 or 3) were equal to 0.41 and 0.57 in AA and EA, respectively.

of the other group. Cg04551776-*AHRR*, cg21161138-*AHRR*, cg03546163-*FKBP5*, cg11660018-*PRSS23*, and cg03152187-*SEPTIN9* associations were found significant only in AA. The effect sizes in AA and EA were very similar (|relative difference| ≤ 0.5), indicating probably limited power to detect these associations in EA participants. Cg05951221-*(ALPG)*, cg25046651-*ADHFE1*, cg26723847-*VPS26B; NCAPD3* and cg18335991-*SEMA7A* were significant only in AA, with opposite directions or large difference of effect sizes (|relative difference| > 2) between AA and EA. Finally, cg14527250-*WNT5B* and cg22749855-*SOCS3* were significant only in EA.

Comparison of significant associations across ancestries and IR measures

We compared the association of the 39 significant CpG sites between AA and EA populations for each of the three IR measures (Table 3 and Figure 1 (TyG-BMI), and Figure S2A (TyG)). Three significant CpG sites associated with TyG were shared in AA and EA participants: cg19693031-*TXNIP*, cg00574958-*CPT1A* and cg06500161-*ABCG1*. Cg00574958-*CPT1A* and cg06500161-*ABCG1* were significantly associated with TyG-BMI in both AA and EA populations. In addition to these two CpG sites, cg11024682-*SREBF1* was also significantly associated with TyG-BMI in both AA and EA.

We compared significant associations of CpG sites across different IR measures in AA and EA separately (Table 4, Figure S2B-E). For all combinations of IR measures, there was at least one overlapping CpG site. Association of DNAm at cg00574958-*CPT1A* was common to the three EWAS of IR in AA participants. Cg19693031-*TXNIP* was shared across HOMA-IR and TyG in AA participants. Cg11024682-*SREBF1* was shared across TyG and TyG-BMI in AA participants. In addition, shared significant associations between TyG and TyG-BMI include cg00574958-*CPT1A* and cg06500161-*ABCG1* in both AA and EA participants.

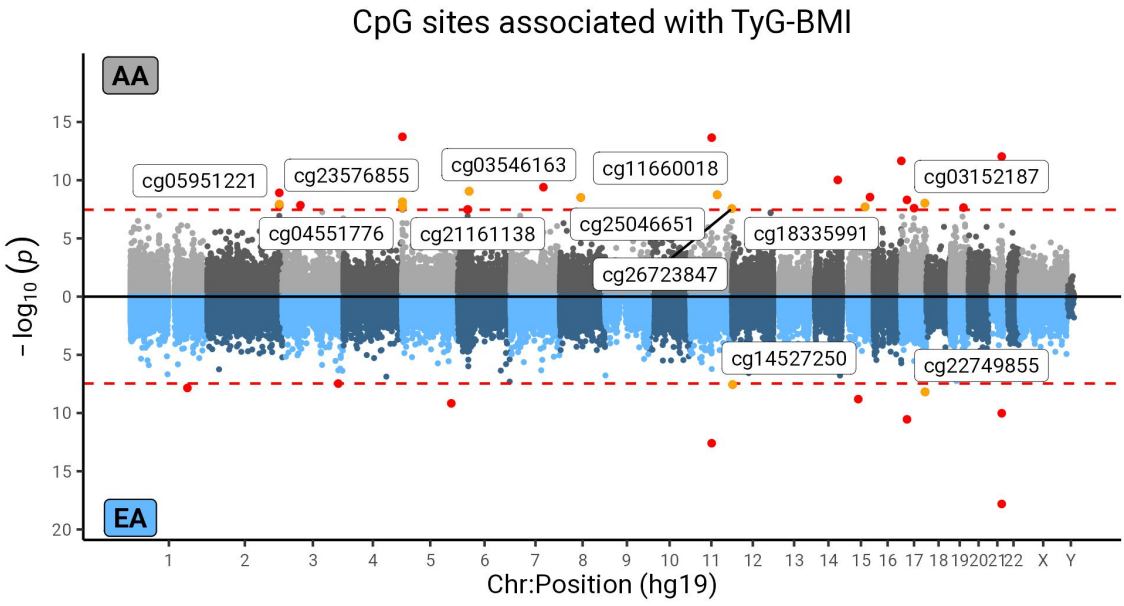

Figure 1. Mirrored Manhattan plots of EWAS of TyG-BMI results in AA and EA populations. Red annotated CpG sites were significantly associated with IR measures at P-value <3.45E–08. AA: African ancestry, EA: European ancestry. Potentially novel CpG sites associated with TyG-BMI were annotated in orange with CpG labels (10 in AA and 2 in EA). Among 27 significant CpG sites in AA (top) and 10 significant CpG sites in EA (bottom), 3 CpG sites were shared between AA and EA (cg00574958, cg11024682, and cg06500161).

Validation of significant associations across population ancestries and IR phenotypes are detailed in the Supplementary Material.

Significant CpG sites previously reported for IR or IR-related traits

Among the 39 significant IR-associated CpG sites, 69% of them ($N = 27$) had been previously reported by IR ($N = 12$) or IR-related traits ($N = 27$) EWAS in the EWAS Catalog and EWAS Atlas databases (Figure S4 and Table S4). Four CpG sites (cg19693031-*TXNIP*, cg00574958-*CPT1A*, cg06500161-*ABCG1*, and cg11024682-*SREBF1*) significant in both AA and EA have been previously reported to be associated with IR or related traits by EWAS. Considering the ancestry of the study population of the published EWAS, 17 out of 27 CpG sites had ancestry-matching IR or IR-related traits EWAS (Table S4). Significant associations detected in AA participants were either

Table 2. List of 12 potentially novel CpG sites associated with IR (quantified by TyG-BMI) in ARIC that have not been previously reported by IR or IR-related traits EWAS.

DNA methylation probe (CpG site)	Gene (Closest gene)	Smoking related?	Drinking related?	African ancestry (AA, N = 1577)			European ancestry (EA, N = 917)		
				Beta	SE	P-value	Beta	SE	P-value
cg05951221	*(ALPG)*	Y	Y	0.061	0.010	**1.18E–08**	0.017	0.015	0.200
cg23576855	*AHRR*	Y	N	0.104	0.016	**7.32E–09**	0.072	0.036	0.029
cg04551776	*AHRR*	Y	N	0.029	0.005	**1.83E–08**	0.019	0.009	0.014
cg21161138	*AHRR*	Y	Y	0.038	0.006	**2.03E–08**	0.026	0.014	0.038
cg03546163	*FKBP5*	N	N	0.075	0.011	**8.93E–10**	0.067	0.017	2.12E–05
cg25046651	*ADHFE1*	Y	N	– 0.012	0.002	**3.06E–09**	0.001	0.004	0.735
cg11660018	*PRSS23*	Y	Y	0.042	0.006	**1.81E–09**	0.028	0.011	0.004
cg26723847	*VPS26B; NCAPD3*	Y	N	– 0.013	0.002	**2.77E–08**	0.001	0.003	0.603
cg14527250	*WNT5B*	N	N	– 0.004	0.012	0.567	– 0.079	0.016	**2.82E–08**
cg18335991	*SEMA7A*	Y	Y	0.024	0.004	**1.96E–08**	0.007	0.008	0.323
cg03152187	*SEPTIN9*	Y	N	– 0.016	0.003	**9.32E–09**	– 0.012	0.003	1.17E–04
cg22749855	*SOCS3*	Y	N	– 0.004	0.005	0.332	– 0.039	0.007	**6.52E–09**

Smoking related? and Drinking related? is Y (yes) if CpG sites have been previously reported associated with smoking and drinking, respectively by EWAS. All the listed CpG sites were significantly associated with TyG-BMI model. Bold: The significant CpG sites at P-value <3.45E–08 (at the 0.05 level after a Bonferroni correction for the number of CpG sites tested and the number of IR measures).

Table 3. Significant IR-CpG associations (at P-value <3.5E–08) in ARIC EWAS across ancestry groups.

				African ancestry (AA)			European ancestry (EA)		
IR measures	CpG site*	Chr:Position (hg19)	Gene	Beta	SE	P-value	Beta	SE	P-value
TyG	cg19693031	1:145,441,552	*TXNIP*	− 0.026	0.003	4.55E−15	− 0.03	0.004	3.15E−16
TyG	cg00574958	11:68,607,622	*CPT1A*	− 0.008	0.001	1.22E−17	− 0.006	0.001	6.94E−11
TyG	cg06500161	21:43,656,587	*ABCG1*	0.017	0.002	3.31E−14	0.024	0.003	9.20E−19
TyG-BMI	cg00574958	11:68,607,622	*CPT1A*	− 0.016	0.002	2.22E−14	− 0.019	0.003	2.55E−13
TyG-BMI	cg11024682	17:17730094	*SREBF1*	0.034	0.005	4.91E−09	0.04	0.007	2.88E−11
TyG-BMI	cg06500161	21:43,656,587	*ABCG1*	0.041	0.005	9.28E−13	0.064	0.008	1.54E−18

*Previously reported associated with IR or IR-related traits by EWAS.

Table 4. Significant IR-CpG associations (at P-value <3.5E–08) in ARIC EWAS by ancestry group and across IR measures.

				HOMA-IR			TyG			TyG-BMI		
Ancestry	CpG site*	Chr:Position (hg19)	Gene	Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value
AA	cg19693031	1:145,441,552	*TXNIP*	− 0.014	0.002	**8.56E−10**	− 0.026	0.003	**4.55E−15**	− 0.011	0.007	0.103
AA	cg00574958	11:68,607,622	*CPT1A*	− 0.004	0.001	**2.63E−09**	− 0.008	0.001	**1.22E−17**	− 0.016	0.002	**2.22E−14**
AA	cg11024682	17:17730094	*SREBF1*	0.006	0.002	4.76E−04	0.018	0.002	**3.04E−15**	0.034	0.005	**4.91E−09**
AA	cg06500161	21:43,656,587	*ABCG1*	0.008	0.002	1.87E−07	0.017	0.002	**3.31E−14**	0.041	0.005	**9.28E−13**
EA	cg00574958	11:68,607,622	*CPT1A*	− 0.002	0.001	0.064	− 0.006	0.001	**6.94E−11**	− 0.019	0.003	**2.55E−13**
EA	cg06500161	21:43,656,587	*ABCG1*	0.005	0.002	0.035	0.024	0.003	**9.20E−19**	0.064	0.008	**1.54E−18**

*Previously reported associated with IR or IR-related traits by EWAS. Bold: The significant CpG sites at P-value <3.45E–08 (at the 0.05 level after a Bonferroni correction for the number of CpG sites tested and the number of IR measures). AA: African ancestry, EA: European ancestry.

previously reported in a different ancestry or in the same ancestry, but with an IR-related trait, not directly IR (Table S4). On the other hand, in EA, not only all significant associations with previously reported IR or IR-related traits EWAS were identified in EA, but also most of the associations were reported with IR traits (Table S4).

Interaction analysis with sex

We examined the interaction effect between IR and sex for the 39 significant IR-associated CpG sites (Table S5). The association between IR and DNAm varied by sex for cg05575921-*AHRR*, cg21161138-*AHRR*, and cg03546163-*FKBP5* at a significance level of 0.05 after Bonferroni correction for the number of IR-associated CpG sites (P-value <1.28E–03).

Sensitivity analysis adjusted for both smoking and drinking status

Among the potentially novel CpG sites ($N = 12$), which have no reported EWAS associations with IR or IR-related traits, 10 of them were previously reported associated with smoking, and four of them were previously reported associated with alcohol consumption, respectively (Table 2). The additional adjustment attenuated the significance of 26 out of 39 associations (P-value ≥3.45E–08), which were mostly associated with TyG-BMI in AA before the adjustment. Four CpG sites showed the largest attenuation (P-value after adjustment > 4.0E–04), three of them being in the *AHRR* gene which is associated with smoking [46] and the other one annotated to a closest gene, *ALPG* (Table S6). Associations with IR for 15 out of the 39 significantly IR-associated CpG sites in the primary EWAS analysis that showed significant or suggestive evidence after additional adjustment for smoking and drinking status are presented in Table S7.

Identification of mQTLs and eQTMs

We assessed cis-acting genetic variants (mQTLs) associated with DNAm at the 12 potentially novel CpG sites detected associated with IR. We identified 1,150 and 976 significant cis-mQTL associations in AA ($p < 7.5E–07$) and EA ($p < 1.3E–06$), respectively (Tables S8–9). The percentage of cis-mQTL variants in the same genes as the ones annotated to the CpG sites was 26% ($N = 294$) and 34% ($N = 328$) in AA and EA, respectively. We observed significant cis-mQTLs at six of the 12 CpG sites tested in EA and six of the 12 CpG sites tested in AA, among which four (cg05951221, cg04551776, cg26723847, cg14527250) were

common among EA and AA. There were 177 mQTLs at three CpG sites (cg05951221, cg26723847, cg14527250) that were shared between AA and EA groups, where most of the shared mQTLs (167 out of 177) had the same direction of effect.

Next, we searched for cis-mQTLs in four publicly available mQTL databases. Most of the cis-mQTL variants were in the same genes as the ones annotated to the CpG sites. In all three mQTL databases of blood samples (Tables S10–S12), we found cis-mQTLs at cg11660018-*PRSS23* with variants in *PRSS23*. Across blood and brain tissues (Tables S10–S13), three CpG sites (cg04551776-*AHRR*, cg11660018-*PRSS23* and cg14527250-*WNT5B*) had mQTLs in both brain (Brain xQTLServe) and blood (GoDMC and BIOS QTL browser), even though mQTLs were not the same in both tissues. DNAm at cg04551776-*AHRR* was reported associated with SNPs in *AHRR* and *SLC9A3* in blood, whereas it was associated with SNPs in *AHRR*, *C5orf55*, *EXOC3*, and *PDCD6* in brain. DNAm at cg11660018-*PRSS23* was found associated with SNPs in *PRSS23* and *ME3* in blood, and with SNPs in *PRSS23* in the brain. DNAm at cg14527250-*WNT5B* was reported associated with SNPs in *WNT5B*, *MIR3649*, *CACNA2D4*, and *ADIPOR2* in the blood, and with SNPs in *WNT5B* in brain.

In addition to mQTLs, we explored cis-eQTMs in the BIOS QTL browser to detect CpG sites whose methylation is associated with gene expression. We found a negative association between methylation and gene expression at cg04551776 (in *AHRR*)-*EXOC3*, cg21161138 (in *AHRR*)-*EXOC3*, and cg18335991 (in *SEMA7A*)-*SEMA7A*.

We further investigated functional annotations of genes annotated to eight out of twelve potentially novel CpG sites that remained significant or suggestive after adjusting for smoking and drinking status (Table S14). Many genes were linked to features related to insulin resistance, glucose metabolism, type 2 diabetes, and obesity. To find IR or IR-related GWAS associations, we also searched for these annotated genes in the GWAS Catalog [45] (Table S14). We found GWAS associations for IR or IR-related traits for genetic variants in the genes annotated to four of the potential novel CpG sites, cg03546163-*FKBP5* (BMI), cg18335991-*SEMA7A* (BMI, blood pressure, beta-aminoisobutyric acid level), cg03152187-*SEPTIN9* (type 2 diabetes, BMI, blood pressure), and cg03152187-*SOCS3* (abdominal adipose tissue volumes). We verified whether there were overlapping mQTLs and eQTLs [43] at these CpG sites. Both in EA and AA groups, cg14527250-*WNT5B* had the same two cis-mQTLs in *WNT5B* that were also eQTLs of *WNT5B* all with the same direction of effect. We also searched for overlapping mQTLs and eQTLs in the publicly available mQTL databases. In the ARIC AA group, cg25046651-*ADHFE1* had seven cis-mQTLs in *ADHFE1* that were also eQTLs of *ADHFE1* all with the opposite direction of effect, cg21161138-*AHRR* had 61 cis-mQTLs in *AHRR* that were also eQTLs of *AHRR* all with the same direction of effect, and cg26723847-*VPS26B;NCAPD3* had 28 mQTLs in *VPS26B* or *NCAPD3* that were also eQTLs mostly with the same direction of effect (86%). In the ARIC EA group, cg23576855-*AHRR* had 66 cis-mQTLs in *AHRR* that were also eQTLs of *AHRR* all with the same direction of effect except for one variant, and cg26723847-*VPS26B;NCAPD3* had 31 mQTLs in *VPS26B* or *NCAPD3* that were also eQTLs with the same direction of effect except for one variant. We also searched for overlapping mQTLs and eQTLs in the publicly available mQTL databases. A cis-acting blood mQTL variant (rs78994380 in *SEMA7A*) [38] at cg18335991-*SEMA7A* was also a blood cis-eQTL of *SEMA7A* (z-score= −5.18, P-value = 2.18E–07) [43]. A cis-acting blood mQTL variant (rs72913417 in *FKBP5*) [40] at cg03546163-*FKBP5* was also detected as a blood cis-eQTL of *FKBP5* (rs72913417, z-score= −4.27, P-value = 1.91E–05) [43]. A blood cis-mQTL variant (rs312824 in *SEPTIN9*) [41] at cg03152187-*SEPTIN9* was also a blood cis-eQTL of *SEPTIN9* (rs312824, z-score = 6.23, P-value = 4.71E–10) [43].

Discussion

In this EWAS, we identified 39 CpG sites associated with three surrogate IR measures, HOMA-IR, TyG, and TyG-BMI, in AA and EA participants from the ARIC study. Using TyG and TyG-BMI, which are more recently proposed IR surrogates compared to HOMA-IR, we identified 12 potentially novel CpG sites that have not been previously reported by EWAS of IR or IR-related

traits. Four CpG sites were identified in both EA and AA and across IR measures (cg19693031-*TXNIP*, cg00574958-*CPT1A*, cg06500161-*ABCG1*, and cg11024682-*SREBF1*). They were previously reported by EWAS of (fasting) glucose (all four CpG sites), triglycerides (all four CpG sites), (fasting) insulin (cg00574958, cg06500161, and cg11024682), and HOMA-IR (cg06500161) in EA populations and IR-related traits EWAS in both AA and EA populations.

As previous EWAS studies of IR were primarily conducted in EA and rarely involved AA individuals (Table S1), our identification of CpG sites that have associations with TyG and TyG-BMI in AA can improve our understanding of the epigenetic mechanisms of IR that may be population-specific. Among 29 significant associations found in AA, 25 of them were detected only in AA, and the other four were also identified in EA.

To find associations between DNAm and IR, we not only used HOMA-IR but also TyG and TyG-BMI which have not been commonly used in EWAS of IR. Even though the sample size for TyG and TyG-BMI was slightly smaller than for HOMA-IR, we detected more associations between DNAm and IR when using TyG and TyG-BMI measures than when analysing HOMA-IR. Both analyses using TyG and TyG-BMI as IR surrogate measures detected associations that were previously reported by IR or IR-related EWAS as well as potentially novel associations. The associations detected between DNAm and TyG-BMI include seven CpG sites with ancestry matching IR traits in the EA group and 12 potential novel CpG sites.

For these potential novel CpG sites, we investigated whether neighbouring genes had biological features related to IR or harboured variants previously associated with IR or IR-related traits. Cg18335991 had a positive association with IR (TyG-BMI) only in AA and was annotated to the *SEMA7A* gene. This gene is related to obesity and hepatic steatosis as it inhibits the adipogenesis of preadipocytes and lipogenesis of mature adipocytes [28] (Table S14). Genetic variants annotated to *SEMA7A* in the GWAS Catalog were associated with BMI and blood pressure (Table S14). A negative association between DNAm at cg18335991 and expression of *SEMA7A* in the blood has been reported [38]. Cg03546163-*FKBP5* was another potentially novel CpG site that was positively associated with TyG-BMI in AA. *FKBP5* gene expression in subcutaneous adipose tissue is known to be associated with IR, glucose and lipid metabolism, adipogenesis, and type 2 diabetes [47] (Table S14). Variants in this gene were associated with obesity-related traits (Table S14). Cg03152187-*SEPTIN9* is a potentially novel CpG site negatively associated with TyG-BMI in AA. The *SEPTIN9* gene is a member of the septin family involved in cytokinesis. This gene has been found to be differentially methylated in pancreatic islets of individuals with type 2 diabetes [48] (Table S14). GWAS of type 2 diabetes, obesity, and blood pressure reported variants associated with those traits in *SEPTIN9* (Table S14). We identified a reticulocyte count associated genetic variant (rs312824 in *SEPTIN9*) [41,49] that is a blood cis-mQTL variant [41] at cg03152187 and a blood cis-eQTL of *SEPTIN9* [43]. A positive causal effect of fasting insulin on red blood cell and reticulocyte counts has been found [50], and IR has been shown to be related to an increased red blood cell count [51]. Further analyses are needed to understand the underlying biological mechanism of these potentially novel associations, particularly the ones identified only in AA participants.

Further functional analysis using DNAm data in different tissues or different omics data can improve our understanding of African ancestry-specific pathogenesis of IR and related diseases. Even though we identified 12 potentially novel CpG sites associated with IR measures, our EWAS in the ARIC study included only two population ancestries and thus our results may not be generalizable to other ancestries. EWAS studies using TyG and TyG-BMI in additional studies and population groups can help confirm the potentially novel CpG sites associated with IR measures that we found in this study. Most publicly available mQTLs databases are restricted to European ancestry, limiting our ability to further explore some of the associations detected in our EWAS. Further EWAS studies conducted in AA populations are thus needed to validate the novel associations found in this study.

Conclusion

In this study, we identified CpG sites associated with three different IR measures and found 12 potentially novel CpG sites that have not been previously reported by EWAS of IR or IR-related traits, most of which were detected in the AA ARIC participants. Additional eQTM and functional analyses are needed to help understand the implication of the potentially novel IR-CpG sites associations found in our study.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

J.B., M.F., and M.L.G. contributed to the acquisition of methylation data and its quality control process. M.B. and M.L.G. contributed to data management. J.S. and C.S. contributed to statistical analyses. J.S. and C.S. contributed to the interpretation of the results. J.S., C.S., E.S., M.F., and A.C.M. contributed to the drafting of the manuscript. All authors contributed to the revision of the manuscript. All authors reviewed and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are not readily available, as access to the DNA methylation dataset from ARIC is restricted but available on request at https://aric.cscc.unc.edu/aric9/researchers/Obtain_Submit_Data. Summary statistics of the main results presented in this study are included in the article/supplementary material. Further inquiries to access the full summary statistics can be directed to the corresponding author.

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