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Gene-specific DNA methylation may mediate atypical antipsychotic-induced insulin resistance

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Objectives: Atypical antipsychotics (AAPs) carry a significant risk of cardiometabolic side effects, including insulin resistance. It is thought that the insulin resistance resulting from the use of AAPs may be associated with changes in DNA methylation. We aimed to identify and validate a candidate gene associated with AAP-induced insulin resistance by using a multi-step approach that included an epigenome-wide association study (EWAS) and validation with site-specific methylation and metabolomics data.

Methods: Subjects with bipolar disorder treated with AAPs or lithium monotherapy were recruited for a cross-sectional visit to analyze peripheral blood DNA methylation and insulin resistance. Epigenome-wide DNA methylation was analyzed in a discovery sample (n = 48) using the Illumina 450K BeadChip. Validation analyses of the epigenome-wide findings occurred in a separate sample (n = 72) using site-specific methylation with pyrosequencing and untargeted metabolomics data. Regression analyses were conducted controlling for known confounders in all analyses and a mediation analysis was performed to investigate if AAP-induced insulin resistance occurs through changes in DNA methylation.

Results: A differentially methylated probe associated with insulin resistance was discovered and validated in the fatty acyl CoA reductase 2 (*FAR2*) gene of chromosome 12. Functional associations of this DNA methylation site with untargeted phospholipid-related metabolites were also detected. Our results identified a mediating effect of this *FAR2* methylation site on AAP-induced insulin resistance.

Conclusions: Going forward, prospective, longitudinal studies assessing comprehensive changes in *FAR2* DNA methylation, expression, and lipid metabolism before and after AAP treatment are required to assess its potential role in the development of insulin resistance.

KEYWORDS
antipsychotics, bipolar disorder, insulin, mediation, methylation

It is estimated that approximately 1–2% of the USA's population has bipolar disorder.[1] Psychopharmacologic treatment of bipolar disorder utilizes several different medication classes, including mood stabilizers, antidepressants and benzodiazepines. The atypical antipsychotics (AAPs) have become an important maintenance pharmacotherapeutic option in the past 10 years.[2] Although the efficacy of AAPs in bipolar disorder has been shown in randomized, placebo-controlled trials, they also carry a significant risk of weight gain, diabetes, metabolic syndrome, and cardiovascular disease.[3] This may be particularly concerning in severe mental illnesses, like bipolar disorder, which have elevated rates of metabolic comorbidity, and, in particular, insulin resistance prior to AAP treatment.[4,5] Physical health may be linked

to psychiatric health. Within bipolar disorder, patients with endocrine abnormalities, in particular insulin resistance, have worse psychiatric outcomes.[6–9] The impact of AAP cardiometabolic side effects on long-term psychiatric outcomes is not known; however, given the connection between cardiometabolic and psychiatric health, as well as the high rates of cardiovascular mortality in bipolar disorder, it is important to understand how these side effects occur.[10] Thus, there is a need to identify the mechanisms by which AAPs increase the risk of insulin resistance so that the beneficial effects of these medications can be used to treat psychiatric symptoms without sacrificing a patient's physical health or compounding the problem of metabolic illness within this vulnerable population.

Pharmacoepigenetics may aid in understanding the complex mechanisms of AAP-induced side effects by examining the interface of medication use and gene expression. Previous work has identified associations between one-carbon metabolism and AAP-induced insulin resistance and metabolic syndrome in bipolar disorder as well as psychopharmacology-specific effects on the epigenome.[11–13] It may be plausible that an epigenetic mechanism underlies these side effects, since the endpoint of one-carbon metabolism is the production of methyl donors that are used for various methylation reactions in the body, including DNA methylation. This hypothesis was supported by identified associations between a global measure of DNA methylation and AAP-induced insulin resistance within bipolar disorder.[14] In the only candidate gene study to date, Moons and colleagues found no associations between AAP-induced metabolic side effects and genetic or epigenetic variability in the insulin-like growth factor 2 (*IGF2*) gene.[15]

The metabolome, which represents all of the small molecules in the cell that arise as a consequence of metabolism, is directed by upstream processes including genetic and epigenetic variability. Although it can be relatively stable, AAPs may have significant effects on the metabolome.[16–19] Metabolomics can be a useful tool in studying AAP-induced side effects due to its ability to represent the echo of complex lifestyles of individuals interacting with genetic and environmental factors such as medications.[20] The changes in the metabolome secondary to AAP-induced insulin resistance may be the functional consequence of epigenetic changes, and therefore combining these two molecular approaches may aid in understanding the pathways involved in AAP-induced insulin resistance.[21,22]

Despite previous work implicating DNA methylation changes in AAP-induced insulin resistance at the global level, it is not known which regions or genes may be differentially methylated in AAP-induced insulin resistance and whether it is a potential mediator of the drug-induced side effect. Within this study, we aimed to identify differentially methylated genes associated with AAP-induced insulin resistance in a multi-step, systematic approach (Fig. S1). In the first step, we employed an epigenome-wide association study (EWAS) to discover a candidate gene (or genes). In the next steps, we validated the discovery finding using site-specific and metabolomics analyses in a separate sample. Finally, within the validation analyses, we tested the hypothesis that AAP-induced insulin resistance is mediated by our candidate gene's DNA methylation. Although completion of the multi-step design was not guaranteed, we detail below our successful identification of a novel candidate gene whose differential methylation may mediate AAP-induced insulin resistance.

1 | METHODS

1.1 | Subject recruitment

Samples and data for both the discovery and validation samples came from an ongoing study on the rates of metabolic syndrome in bipolar disorder and schizophrenia.[11] Subjects were included if they had a Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR) diagnosis of bipolar I disorder via the structured clinical interview for DSM diagnoses (SCID) and currently had been treated with an AAP or lithium monotherapy for at least 3 months.[23] Subjects were excluded if they had a documented metabolic disturbance (e.g., obesity, diabetes or cardiovascular disease) prior to starting their AAP or lithium therapy, were currently treated with medications for diabetes, or had an active substance abuse diagnosis (smoking was allowed). Subjects underwent a medical and medication history, and anthropometric, vital sign, psychiatric symptom, and insulin resistance [homeostatic model assessment of insulin resistance (HOMA-IR)] measurements.[24] A fasting (8–10 h) blood draw within 3 h of the subject's normal time of awakening was obtained for epigenetic and metabolomics assessment. Current smoking was defined as smoking five or more cigarettes per day. For the purposes of description, AAP use was stratified based on a general metabolic side effect risk profile to include the following groups: Group 1 included olanzapine and clozapine, which have the highest propensity to cause metabolic side effects, Group 2 included quetiapine and risperidone, which carry a moderate risk of metabolic side effects, and Group 3 included aripiprazole and ziprasidone, which are considered to have the lowest risk of all AAPs of causing metabolic side effects. Such categorization (e.g., low, intermediate and high risk) has been used in previous studies and is supported by meta-analyses and consensus expert opinion.[25–29] All subjects provided informed consent before participating and the study was approved by the University of Michigan Medical Institutional Review Board (UM-MED IRB).

1.2 | Discovery using an EWAS

From the above cohort, 48 subjects currently treated with AAPs were randomly chosen for the discovery step. Genomic DNA was obtained from whole blood, bisulfite converted, and submitted to the University of Michigan DNA sequencing core for analysis using the Illumina Human Methylation 450K BeadChip (San Diego, CA, USA).[30] The core returned raw image (IDAT) files for analysis by the investigators.

1.3 | Validation: site-specific epigenetic analysis

Validation of the top gene-related finding from the EWAS (detailed in the Results) occurred in the validation sample using site-specific methylation with pyrosequencing. The validation sample (n = 72 on

AAP and lithium monotherapy) was chosen from the same pool of samples described in the recruiting process above; however, there was no overlap. Primers were created to measure the methylation of the Chr12:29302232 site (GR37/HG19) identified from the EWAS using the Qiagen Assay Design 2.0 software (Germantown, MD, USA) (Table S1). Duplicate reactions were run on a Qiagen Pyrosequencer MD96[31] and duplicates resulting in a coefficient of variation > 2% methylation difference were discarded. Two samples were removed from validation analysis due to unreliable methylation results. Percent methylation at Chr12:29302232 was standardized using a six-point standard curve.[32]

1.4 | Validation: metabolomics analysis

An untargeted metabolomics analysis in the validation sample was conducted using the Michigan Regional Comprehensive Metabolomics Resource Core (MRC)[2] at the University of Michigan, as described elsewhere.[33] The core processed all samples and resulting features were stored and further processed using in-house developed software and finally returned as a single, normalized (via internal standards), and pre-processed (auto-scaled, log-transformed) data set. The core assisted in narrowing the returned data set to metabolites related to our discovery gene findings using their in-house pathway software as well as commercially available platforms. Metabolites were chosen if they were found in phospholipid pathways (this pathway was chosen based on the discovery EWAS finding) by entering metabolite identifiers into Kegg Reaction, Metaboanalyst and Metscape Databases.

1.5 | Statistical analysis

Demographic and clinical characteristics are described using means with standard deviation or percentages. The discovery and validation samples were compared using Student *t*-tests for continuous variables and chi-square or Fisher's exact tests for categorical data. Raw IDAT data files from the Illumina 450K BeadChip were downloaded from the DNA sequencing core and processed using available bioinformatics pipelines in R Statistical software 3.1.[34,35] These pipelines included the CHAMP, Minfi and COMBAT R packages.[36–39] No samples were removed due to missing data points. Cellular composition was inferred by the method of Houseman and colleagues using the FlowSorted. Blood.450k R package in order to be included as a covariate in the analysis, as it has been shown that cellular heterogeneity can be an important confounder in EWAS and particularly in studying medication effects in bipolar patients taking medications.[12,40,41] To assess the relationship between HOMA-IR and 450K methylation, differentially methylated probes were identified using a linear model ('lm' in Limma package) with the covariates of age, race, gender, AAP group, smoking, folate, body mass index (BMI), and cellular composition.[42] Covariates of age, race, gender, BMI, folate, and smoking were chosen based on our previous work and from work showing that they may be considered biologically relevant predictors of methylation status in addition to HOMA-IR.[14,43] Furthermore, individual antipsychotics have different propensities to cause insulin resistance, and therefore the three AAP groups listed above were also included in the model. The use of linear modeling with Limma and its benefits in analyzing microarray data have been described elsewhere [44] and our modeling improved the overall fit (see QQ plot in Fig. S2). Correction for multiple testing was performed using the Bonferroni method with an epigenome-wide significance level of $P < 1.1 \times 10^{-7}$ (0.05/450 000 sites).

For the validation analyses, the correlation between CpG methylation (at the site identified from the EWAS) and HOMA-IR was analyzed in the validation sample using linear regression.[32] Covariates for linear regressions were chosen based on the literature, previous results (as described above), and the 10% rule to keep covariates that change the association between independent and dependent variables of interest by more than 10% when removed. Cellular composition data were not available for the validation sample and thus could not be included as a covariate. A *P*-value of < .05 was considered significant for the site-specific validation analyses. All genomic coordinates in this study are described using the Human Genome Version 19 (HG19) (http://genome.uesc.ed).

Metabolite data were analyzed by linear regression to assess their association with Chr12:29302232 methylation. Dependent variables were the relative metabolite levels. Covariates were chosen in the same manner as described above for the site-specific analyses. A total of 13 metabolites were analyzed, and a false discovery rate (using the online tool from http://users.ox.ac.uk/~npike/fdr/) corrected *P*-value of < .05 was considered statistically significant.

Finally, to test the hypothesis that AAP-induced insulin resistance may be explained by medication-induced changes in the candidate gene's methylation, we conducted a mediation analysis. We first analyzed the association of AAP use with our candidate gene's methylation by evaluating the effect of medication (AAP or lithium monotherapy; Fig. S1, Step 4). If this association was significant, we then conducted a mediation analysis according to the method of Ditlevsen and colleagues.[45] An overview of the mediation analysis is provided in Fig. S3.

2 | RESULTS

2.1 | Population characteristics

Forty-eight subjects with bipolar I disorder currently treated with an AAP were included in the discovery cohort. A total of 72 subjects with bipolar I disorder treated with AAPs (*n* = 54, 75%) and lithium monotherapy (*n* = 18, 25%) were included in the validation analyses (Table 1). The sets were comparable in terms of demographic and clinical variables and did not significantly differ based on age, race, HOMA-IR or medication use (all *P* > .07). The validation sample did have a higher percentage of female participants (*P* = .08), African American participants (*P* = .22), and a higher BMI (*P* = .09).

2.2 | Discovery sample EWAS

After correction for multiple testing, only the top two hits, cg25329211 and cg10171063, remained significant from the EWAS analysis based on HOMA-IR. Probe cg25329211 is located in an intergenic shelf region on chromosome 2 and probe cg10171063 is located within a

TABLE 1 Demographic and clinical characteristics of discovery and validation samples

	Discovery sample (n = 48)	Validation sample (n = 72)
Age, years, mean ± SD	45.1 ± 11.2	42.3 ± 12.1
% female	54	69.4
Race/ethnicity, % Caucasian; % African American	94; 4	80; 11
BMI, kg/m^2, mean ± SD	29.7 ± 6.29	32.3 ± 9.19
HOMA-IR, mean ± SD	6.53 ± 4.92	5.24 ± 2.96
% smoker	27	32
Folate, ng/ml, mean ± SD	18.9 ± 5.98	18.7 ± 6.00
% Group 1 antipsychotic	16	11
% Group 2 antipsychotic	65	40
% Group 3 antipsychotic	19	24
% lithium monotherapy	–	25

No significant differences were identified between the discovery and validation cohorts. Race/ethnicity is categorized as 'Caucasian', 'African American', and 'Other'. Atypical antipsychotic use was stratified by metabolic risk profile. *Group 1* antipsychotics included olanzapine and clozapine, which have the highest risk to cause metabolic side effects. *Group 2* antipsychotics included quetiapine and risperidone, which carry a moderate risk, and *Group 3* antipsychotics included aripiprazole and ziprasidone, which have the lowest risk to cause metabolic side effects. SD = standard deviation; BMI = body mass index; HOMA-IR = homeostatic model assessment of insulin resistance.

gene called fatty acyl CoA reductase 2 (*FAR2*) on chromosome 12 at position 29302232 (herein referred to as Chr12:29302232 methylation). The *FAR2* enzyme catalyzes the rate-limiting reduction of fatty acyl CoAs to fatty alcohols.[46] The top 10 differentially methylated probes from the EWAS analysis are given in Table 2. A Manhattan plot for the EWAS analysis can be found in Figure 1. Although not significant after Bonferroni correction, three additional probes within the same CpG island (chr12:29302035–29302954) of *FAR2* were in the top 10 most significant sites associated with HOMA-IR.

2.3 | Validation sample: replication of *FAR2* methylation association with insulin resistance

Due to the enrichment of the *FAR2* gene in the top EWAS results, and because probe cg10171063 was the only statistically significant hit located in a gene coding region, we aimed to replicate the association of decreased methylation or hypomethylation at Chr12:29302232 (cg10171063) with increased HOMA-IR (indicating an insulin-resistant state) in an additional sample of subjects with bipolar disorder assessed for insulin resistance and currently treated with AAPs or lithium monotherapy. The average detected methylation in the validation sample at this site was 58.3 ± 16.1 (mean ± standard deviation) and the association of decreased Chr12:29302232 methylation and increased HOMA-IR was replicated (crude beta = −0.08, crude P = .0016, adjusted beta = −0.06, adjusted P = .007) while adjusting for age, race, gender, BMI, and smoking status (Table 3). Use of the same model, while stratifying the validation sample based on medication use (AAP or lithium monotherapy), only showed this negative association between Chr12:29302232 methylation and HOMA-IR in AAP-treated subjects (crude beta = −0.09, crude P = .0047, adjusted beta = −0.07, adjusted P = .018), while no association was observed in subjects treated with lithium (crude beta = −0.005, crude P = .9, adjusted beta = −0.008, adjusted P = .8). Although not done in our primary validation analyses due to a limited sample size, stratifying the sample by AAP metabolic risk (i.e., low, medium and high) showed that within all three groups the same negative correlation between Chr12:29302232 methylation and HOMA-IR was observed. The relationship between Chr12:29302232 hypomethylation and higher HOMA-IR in the olanzapine and clozapine group (high metabolic risk) did not reach statistical significance (beta = −0.11, P = .06) which may be due to the small sample size in this group. The medium-risk group

TABLE 2 Top 10 differentially methylated probes from EWAS analysis based on HOMA-IR

CpG probe	Chromosome	Gene	Base pair	Feature	Log_2 fold change	Original P-value	Bonferroni corrected P-value
cg25329211	2	NA	64375257	IGR–Shelf	−0.004	4.56E-08	.01
cg10171063	12	*FAR2*	29302232	Exon 1–Island	−0.005	6.98E-08	.02
cg12503835	8	*TSNARE1*	143324660	Body–Open Sea	−0.02	1.69E-07	.06
cg04488853	11	*CCKBR*	6293288	3′ UTR–Shore	−0.02	2.49E-07	.08
cg10902825	12	*FAR2*	29302179	Exon 1–Island	−0.005	2.57E-07	.09
cg11390073	12	*FAR2*	29302515	Intron 1– Island	−0.009	3.00E-07	.10
cg24677744	12	*FAR2*	29302016	Exon 1–Island	−0.008	3.01E-07	.10
cg20697767	17	*RPRML*	45056325	Exon 1–Island	0.02	3.41E-07	.12
cg21725878	12	NA	132956918	IGR–Island	−0.01	3.86E-07	.13
cg15076218	1	*PIK3R3*	46599002	TSS1500–Island	0.01	4.02E-07	.14

The table lists the top 10 differentially methylated probes (CpG probes) from the epigenome-wide association study (EWAS) analysis based on homeostatic model assessment of insulin resistance (HOMA-IR) while adjusting for age, race, gender, atypical antipsychotics group, smoking, folate, body mass index, and cellular composition. Location, gene (if applicable), feature of probe and log_2 fold change are listed along with original and Bonferroni adjusted P-values. The top two hits remained significant after multiple testing correction.

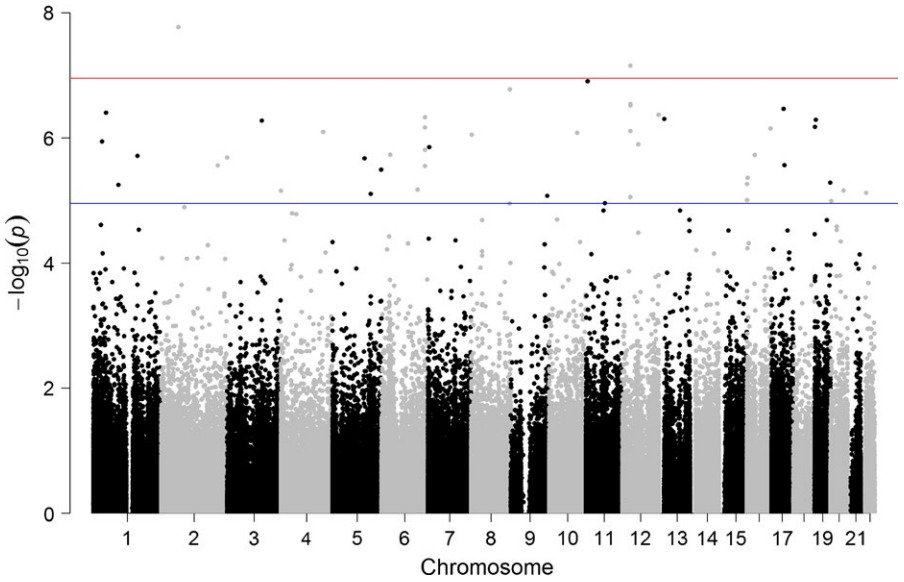

FIGURE 1 A Manhattan plot of the *P*-values based on homeostatic model assessment of insulin resistance (HOMA-IR) while adjusting for age, race, gender, antipsychotic type, smoking, folate, body mass index (BMI) and cellular composition. The *x*-axis is broken down by chromosome and the *y*-axis is the $-\log_{10}$ *P*-value. The two probes that met the epigenome-wide association study (EWAS) significance level (upper horizontal line) were in chromosome 2 (probe cg25329211) and chromosome 12 (probe cg10171063). The lower horizontal line is the suggestive EWAS significance threshold cutoff. Note the two hits above the significance threshold of 1.1×10^{-7} in chromosomes 2 and 12 correspond to the top two hits in Table 2

TABLE 3 Validation analyses

Dependent variables	CHR12:29302232 crude beta coefficients	Crude *P*-value	CHR12:29302232 adjusted beta coefficients	Adjusted *P*-value
HOMA-IR	−0.08	.0016*	−0.06	.0070*
Arachidonoylglycerophosphoethanolamine	15762.91	.36	11314.09	.57
Hexadecanedioate	0.0069	.47	0.0017	.86
Linoleoylglycerophosphoethanolamine	−248848.80	.045*	−393919.30	.033*
Octadecanedioate	0.016	.0395*	0.010	.21
Oleoylglycerophosphoethanolamine	19886.00	.76	−2079.11	.98
Palmitoleic acid	0.0031	.42	0.004	.41
Palmitoleoylglycerophosphocholine	−0.018	.047*	−0.020	.049*
Palmitoyl sphingomyelin	−0.015	.035*	−0.018	.028*
Palmitoylcarnitine	0.0018	.84	0.0033	.74
Palmitoylplasmenylethanolamine	0.004	.41	0.003	.62
Palmitoylglycerophosphoinositol	−0.0013	.88	−0.0066	.50
Stearoylglycerophosphocholine	−0.0036	.57	−0.0021	.82
Stearoylglycerophosphoethanolamine	−0.0040	.62	−0.0089	.34

Validation analyses investigated associations between CHR12:29302232 methylation and insulin resistance as measured by homeostatic model assessment of insulin resistance (HOMA-IR) and were adjusted for age, race, gender, body mass index (BMI), and smoking status. Additional functional analyses investigated associations between Chr12:29302232 and an untargeted metabolomics set related to phospholipid metabolites, adjusting for age, race, gender, BMI and folate levels. The units of each metabolite are relative arbitrary units established by the metabolomics core processing methods. Presented *P*-values for the functional metabolite analyses were corrected for multiple testing using the false discovery rate method (a total of 13 metabolites/tests).
*$P < .05$.

and low-risk group still had significant negative correlations between Chr12:29302232 methylation and HOMA-IR (beta = −0.09, $P = .047$; and beta = −0.07, $P = .014$, respectively).

2.4 | Validation sample: metabolomics analyses for functional effects of *FAR2* methylation

Our top gene-related finding from the EWAS analysis identified differential methylation of a lipid enzyme gene associated with HOMA-IR (*FAR2*). We restricted our metabolomics analyses in the validation sample to identified metabolites related to pathways that could be associated with *FAR2* in order to reduce Type I error. A total of 13 metabolites related to these pathways were identified from the known subset of metabolites. These metabolites, as well as crude and adjusted beta values and the *P*-values, for the regressions with Chr12:29302232 methylation are listed in Table 3. The levels of linoleoylglycerophosphoethanolamine, palmitoleoylglycerophosphocholine and palmitoylsphingomyelin were negatively correlated to Chr12:29302232 methylation, meaning that hypermethylation at this site was associated with lower metabolite levels.

2.5 | Validation sample: testing of AAP effects on *FAR2* methylation and HOMA-IR

In the next step of our approach (Fig. S1), we investigated the effect of AAP use on *FAR2* methylation and HOMA-IR. A linear regression adjusting for age, race, and gender confirmed the association between AAP use and *FAR2* methylation (crude beta = −5.27, *P* = .037, adjusted beta = −5.28, adjusted *P* = .040). Our data also confirmed the well-studied and known effect of AAP use on insulin resistance. A regression adjusting for age, gender, race, BMI and smoking identified a significant association between AAP use and HOMA-IR (crude beta = 0.922, *P* = .022, adjusted beta = 0.751, adjusted *P* = .028).

2.6 | Validation sample: mediation analyses

A mediation analysis aids in understanding to what degree, if any, *FAR2* methylation may be the mediating factor between AAP use and HOMA-IR. The coefficients from sequential modeling described above indicated that there were significant associations between (i) AAP use and HOMA-IR, (ii) AAP use and *FAR2* DNA methylation, and (iii) *FAR2* methylation and HOMA-IR. The association between AAP use and HOMA-IR was not significant when accounting for *FAR2* DNA methylation, suggesting a complete mediation effect by *FAR2* methylation. Calculations using the crude beta coefficients from these consecutive regressions (see Fig. S3) estimated a percent mediation of 40%, indicating that 2/5 of the risk of AAP-induced insulin resistance may be mediated by hypomethylation at the *FAR2* gene. Thus, the direct mediation effect is 60%.

3 | DISCUSSION

To our knowledge, this is the first study to use an epigenome-wide platform to investigate epigenetic associations with an AAP-induced metabolic side effect. Two differentially methylated probes were identified in the discovery set, one of which was found within a gene with possible biological relevance (i.e., lipid metabolism) to insulin resistance. This methylation site, within the *FAR2* gene on chromosome 12 at position 29302232, was successfully replicated in a separate, validation sample using site-specific methylation analyses and found to be a mediator of the association between AAP use and insulin resistance.

3.1 | Findings from EWAS and methylation validation

The top hit, probe cg25329211 (Chr2: 64375257), is located within an intergenic shelf region. The significance of such methylation sites is not fully known. However, these locations may be important for regulating alternative promoters, preventing unneeded expression from intergenic regions, and for splicing events.[47,48] Further work is needed to investigate the relevance of this finding and its relationship to AAP-induced insulin resistance, if any. It may be possible that this site could serve as a biomarker regarding the risk for AAP-induced insulin resistance despite its current unknown functional significance.

The second CpG site from the EWAS that remained significant after correction for multiple testing was located at Chr12:29302232 (cg10171063). Given the association of this site with a gene, we chose to focus our validation work here. We were able to confirm the association of hyopmethylation at this CpG site with insulin resistance as measured by HOMA-IR in our validation sample. The methylation site of Chr12:29302232 is found within a CpG Island of exon 1 of the *FAR2* gene (also known as the male sterility domain-containing protein 1 [*MLSTD1*] gene) within 150 bp of a transcription start site. This gene encodes a peroxisomal protein that utilizes fatty acyl CoA substrates for conversion to fatty alcohols.[46] The production of fatty alcohols is the rate-limiting step to the downstream formation of wax monoesters and ether phospholipids. Ether phospholipids include both an ether bond to an alkyl moiety and an ester bond to a fatty acid, and are thought to be important constituents of cell membranes and possibly cell signaling.[49] One of the most common classes of ether lipids is called plasmalogens, which contain a phospholipid with a glycerol chain attachment.[50,51] Plasmalogens are found within many human tissues, with particularly high concentrations in the central nervous system and cardiovascular system, accounting for 15–20% of total phospholipid content in cell membranes.[50,52] Plasmalogens may have an important role in the cardiometabolic health of certain populations.[53,54]

3.2 | Phospholipid changes and AAP use

Changes in phospholipids including the plasmalogens have been identified following AAP treatment in several metabolomics studies in schizophrenic subjects.[16–19,33] In two lipidomic studies, by Kaddurah-Doak and McEvoy and colleagues, significant elevations in phospholipid levels were found following AAP treatment in several different populations of schizophrenic patients including first episode, treatment naïve, and recurrent episode patients compared to controls. Within both studies, increases in a phospholipid, phosphatidylethanolamine, were identified after treatment with AAPs. Xuan and colleagues found that *myo*-inositol, which is required for the synthesis of membrane inositol phospholipids, was a statistically significant metabolite that defined AAP treatment response in their untargeted metabolomics study of risperidone effects in schizophrenic subjects. Finally, Oresic and colleagues[55] conducted a lipidomic investigation in twin pairs discordant for schizophrenia (74% of twins with schizophrenia were taking an AAP) and found varying levels of lipids depending on the subspecies being analyzed. With their study, they specifically identified increases in sphingomyelins in schizophrenic subjects, which may support our finding of an association between palmitoylsphingomyelin and Chr12:29302232 methylation. Finally, although the difference was not statistically significant in their study, Oresic identified higher levels of ether phospholipids, which are directly controlled by the activity of our *FAR2* candidate gene, in schizophrenic subjects compared to their discordant twins and healthy controls.

Within our study, insulin resistance was associated with hypomethylation of *FAR2*. Our study identified possible functional effects of Chr12:29302232 methylation using an untargeted metabolomics analysis of phospholipid-related metabolites. We identified an inverse

relationship between Chr12:29302232 methylation and three metabolites, linoleoylglycerophosphoethanolamine, palmitoleoylglycerophosphocholine and palmitoylsphingomyelin, where hyopmethylation was associated with higher metabolite levels. This relationship may follow the general notion that decreased methylation of a gene results in increased expression of downstream product associated with that gene or vice versa. It may further suggest that methylation in this particular CpG island of *FAR2* may be important for *FAR2* gene regulation. It may also be plausible that differences in methylation of rate-limiting enzymes like that of *FAR2* could have substantial effects on the lipid balances occurring in the body.

The plasmalogen phospholipids have been characterized as polyunsaturated fatty acid (PUFA) 'sinks' due to their higher likelihood of having linkage to both the n-3 and n-6 PUFAs compared to the diacyl alternative.[56] Our metabolites included two phospholipids with PUFA attachments (arachidonoylglycerophosphoethanolamine and linoleoylglycerophosphoethanolamine). Higher relative linoleoylglycerophosphoethanolamine levels were found to be significantly associated with lower Chr12:29302232 methylation. The intersection of PUFA and phospholipid metabolism continues to be a theme in the literature regarding both positive and negative AAP outcomes in bipolar disorder and schizophrenia [16,18,19,55,57] and further exploration may be warranted.

3.3 | DNA methylation as a mediator of AAP use and insulin resistance

Finally, our work showed a mediating effect by DNA methylation at *FAR2* on AAP-induced insulin resistance. This may give preliminary and partial support to our overall hypothesis that AAPs cause changes in DNA methylation at particular locations in the genome which subsequently alter RNA, protein and metabolite regulation to cause insulin resistance. We speculate that the lowering of *FAR2* methylation by AAPs leads to increased production of certain lipid species, leading to a lipid imbalance and increased insulin resistance. Lipid imbalances have been implicated in insulin resistance pathophysiology.[58] Ultimately, the molecular cause of AAP-induced insulin resistance is likely multifactorial and may include several different pathways. It could also be highly dependent on the particular population being studied. Indeed, genetic variation of lipid-metabolizing enzymes from other pathways has been linked to AAP-induced insulin resistance.[59] Our work and that of others has suggested that AAPs may have significant effects on the epigenome which could have implications for both therapeutic and adverse effects.

3.4 | Strengths and limitations

The findings from this study should be interpreted cautiously in light of two main limitations, namely (i) a limited sample size and (ii) lack of longitudinal sampling. Psychiatric polypharmacy is well known in bipolar disorder and, although we were able to overcome this in our sample, it did limit the sample size, which reduces power when correcting for multiple comparisons. Previous work has identified an optimal sample size of at least 112 cases and controls to achieve 80% power in detecting methylation differences in epigenome-wide studies.[60] The current study did not reach this suggested sample size and it did not include controls and thus could have insufficient power to detect true findings. Additionally, the lack of longitudinal samples does not allow us to infer causal changes but only single time-point methylation and metabolite associations in a population already on AAPs or lithium for at least 3 months. Such a study design carries treatment length and dosage variability that should be taken into account when interpreting the results. Thus, replication in longitudinal formats and other AAP-treated populations should be pursued to investigate the validity of our identified gene as well as other top hits from our study and/or other candidate pathways involved in AAP-induced insulin resistance.

A few other limitations should be considered. We did not have the ability to assess changes in *FAR2* gene expression (with RNA) in relation to AAP-induced insulin resistance as RNA was not available. We also only validated a single DNA methylation site and studies in the future should expand the methylation analyses to include other areas of the *FAR2* gene. Our metabolite data only measured relative levels, and so the lipidomic pathways that may be influenced by this gene's methylation require further, targeted exploration. Finally, our work also included both obese and nonobese subjects. BMI is a well-known risk factor of insulin resistance and this was seen in our study samples (data not shown). Nevertheless, when analyses were adjusted for BMI, our findings were still significant. Additionally, we describe the effects of obesity on Chr12:29302232 methylation and metabolite levels in Table S2 for descriptive purposes; however, detected effects were minimal. Obesity remains a significant independent predictor of insulin resistance and so future studies will need to separate the effects of AAP-induced obesity and insulin resistance on gene methylation and metabolite changes with either prospective studies or studies in nonobese populations.

This study had several strengths, including an epigenome-wide analysis coupled with validation analyses that utilized site-specific methylation and metabolomics data in two samples of subjects with bipolar disorder with well-characterized medication regimens. Within the top ten differentially methylated genes associated with HOMA-IR from our EWAS analysis, four were found within the same CpG island of the *FAR2* gene and all were found to have lower methylation with higher HOMA-IR. This should be interpreted cautiously as only the top two genes from this list remained significant after multiple testing corrections, but our unbiased discovery approach followed by validation does strengthen this candidate gene finding. Although the sample size was limited and cross-sectional, the ability to replicate our finding and the possible biological relevance of the *FAR2* gene do strengthen the findings in their present form. We cannot rule out a reverse causation effect on our identified gene methylation, meaning that insulin resistance causes changes in gene methylation. However, our multi-step approach in the validation sample of identifying a mediation effect reduces the chance of reverse causation. Finally, this work was performed in the peripheral blood, which is heterogeneous in cell type. Our EWAS analysis used available strategies to control for the heterogeneity but we were unable to control for this in our validation sample, where the DNA had already been collected. Due to this heterogeneity, future work will need to translate the peripheral blood findings to a

target tissue. There are several target tissues that may be good candidates for investigating the epigenetic and molecular mechanisms of AAP-induced insulin resistance which our group is currently examining.

4 | CONCLUSIONS

This study identified and validated a differentially methylated CpG site in a novel gene associated with AAP-induced insulin resistance. We were able to identify a mediating effect of this CpG site on AAP-induced insulin resistance as well as possible functional effects of lipid metabolites. To date, we still do not fully understand the precise mechanism of AAP-induced insulin resistance. Identification of the mechanism of AAP-induced insulin resistance could lead to future personalized intervention strategies such as biomarkers that will allow us to screen patients for the risk of becoming insulin resistant or potentially allow us to design drug therapy to prevent AAP-induced insulin resistance itself. The interventions based on such knowledge will allow clinicians to utilize AAPs for their beneficial, psychiatric effects, while minimizing or removing the detrimental effects on cardiometabolic and physical health which will ultimately improve a psychiatric patient's morbidity and mortality. Going forward, targeted, prospective epigenetic analyses in candidate insulin-sensitive tissues are needed to fully understand the interaction of genes and medications in the severely mentally ill.

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DISCLOSURES

The authors of this paper do not have any commercial associations that might pose a conflict of interest in connection with this manuscript.

AUTHOR CONTRIBUTIONS

KJB conceived and designed the study, prepared samples for analysis, conducted site-specific assays, performed statistical analyses, and wrote the article. JMG assisted in the epignome-wide analyses, site-specific methylation analyses, statistical analyses, and manuscript writing. DCD assisted in statistical analyses, interpretation, and manuscript writing. VLE provided samples, assisted in study design, provided equipment for site-specific methylation analyses, and assisted in interpretation of results and manuscript writing.

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