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Integrative Genetic Variation, DNA Methylation, and Gene Expression Analysis of Escitalopram and Aripiprazole Treatment Outcomes in Depression: A CAN-BIND-1 Study

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ABSTRACT

Introduction Little is known about the interplay between genetics and epigenetics on antidepressant treatment (1) response and remission, (2) side effects, and (3) serum levels. This study explored the relationship among single nucleotide polymorphisms (SNPs), DNA methylation (DNAm), and mRNA levels of four pharmacokinetic genes, *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1*, and its effect on these outcomes.

Methods The Canadian Biomarker Integration Network for Depression-1 dataset consisted of 177 individuals with major depressive disorder treated for 8 weeks with escitalopram (ESC) followed by 8 weeks with ESC monotherapy or augmentation with aripiprazole. DNAm quantitative trait loci (mQTL), identified by SNP-CpG associations between 20 SNPs and 60 CpG sites in whole blood, were tested for associations with our outcomes, followed by causal inference tests (CITs) to identify methylation-mediated genetic effects.

Results Eleven *cis*-SNP-CpG pairs ($q < 0.05$) constituting four unique SNPs were identified. Although no significant associations were observed between mQTLs and response/remission, *CYP2C19* rs4244285 was associated with treatment-related weight gain ($q = 0.027$) and serum concentrations of ESC_{adj} ($q < 0.001$). Between weeks 2-4, 6.7 % and 14.9 % of those with *1/*1 (normal metabolizers) and *1/*2 (intermediate metabolizers) genotypes, respectively, reported ≥ 2 lbs of weight gain. In contrast, the *2/*2 genotype (poor metabolizers) did not report weight gain during this period and demonstrated the highest ESC_{adj} concentrations. CITs did not indicate that these effects were epigenetically mediated.

Discussion These results elucidate functional mechanisms underlying the established associations between *CYP2C19* rs4244285 and ESC pharmacokinetics. This mQTL SNP as a marker for antidepressant-related weight gain needs to be further explored.

Introduction

Antidepressants are effective at relieving the symptoms of major depressive disorder (MDD), which has been ranked by the World Health Organization as the leading contributor to global disability [1]. However, there is significant interindividual variability in antidepressant treatment response and adverse effects, frequently resulting in initial treatment failure [2–4]. Therefore, there exists a clear need to identify clinically meaningful predictors of outcomes earlier during the initiation of antidepressant pharmacotherapy [5–7].

Polymorphisms in genes related to the pharmacokinetics of some antidepressants, including hepatic drug-metabolizing cytochrome P450 enzymes, CYP2C19 and CYP2D6, and the transmembrane P-glycoprotein (P-gp) efflux pump of the blood-brain barrier, were shown to contribute to the inter-individual variability in blood drug and metabolite concentrations [8–10]. Variants of these pharmacokinetic genes may also contribute to antidepressant treatment response and adverse reactions; however, evidence for these associations has been mixed [9, 11, 12]. This is because genetic variations between individuals only explain a modest proportion of interindividual differences in antidepressant pharmacokinetics and treatment outcomes [13]. Epigenetic mechanisms, which mediate the interaction between genetic and environmental factors, have become increasingly recognized as promising targets for biomarker development [13, 14]. DNA methylation (DNAm), involving the covalent addition of a methyl group to the fifth carbon of the cytosine pyrimidine ring that precedes a guanine nucleotide within genes (CpG sites), is a relatively stable epigenetic modification regulating gene expression which has been widely studied [15]. Thus far, research on the potential clinical utility of DNAm biomarkers as predictors of antidepressant outcomes is currently in its infancy and has been inconclusive [16].

Characterizing the interplay between genetic, DNAm, and gene expression variability through quantitative trait loci (QTL) analyses is an approach that has the potential to increase understanding of the mechanisms contributing to clinical outcomes of antidepressants and lead to biomarker discovery. Methylation quantitative trait loci (mQTLs) and expression quantitative trait loci (eQTLs) are single nucleotide polymorphisms (SNPs) that influence levels of DNAm and gene expression of one or more genes, respectively [17–19]. Accumulating evidence demonstrates an association between specific mQTLs and the risk and phenotypes of affective disorders, including MDD [20, 21], bipolar disorder [22], and neuroticism [23]. However, there has been a lack of research on identifying mQTLs and eQTLs that influence outcomes of treatment with antidepressants and which can reliably be used as markers for medication selection and/or dosing.

Therefore, the objective of our present study was to investigate the effects of the complex relationship between genetic and epigenetic variation across four genes involved in the pharmacokinetics of escitalopram (ESC), *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1*, on treatment outcomes and drug serum levels. To carry out our study objective, we identified mQTLs of these genes and determined their associations with our outcomes of interest: (1) treatment response and remission, (2) side effects, and (3) serum concentrations of drug, its metabolites, and metabolite-to-drug ratio in the CAN-BIND-1 sample of MDD patients treated with sequential ESC monotherapy or adjunctive therapy with an antipsychotic, aripiprazole (ARI). We also explored the effect of significant mQTL SNPs on gene expression using eQTL analysis and assessed the role of DNAm in mediating eQTL effects.

Methods

Participants

This is a secondary analysis of data from the Canadian Biomarker Integration Network in Depression Study-1 (CANBIND-1, ClinicalTrials.gov Identifier: NCT01655706), a Canada-wide multi-centre initiative for the discovery of biomarkers of MDD treatment re-

sponse, which has been described in detail elsewhere [24, 25]. Participants with MDD were recruited between August 2013 and December 2016 from six Canadian outpatient centres. Inclusion criteria: (1) age 18-60 years, (2) MDD diagnosis confirmed using the Mini-International Neuropsychiatric Interview [26], (3) a Montgomery-Asberg Depression Rating Scale (MADRS) [27] score of ≥ 24 (indicating the presence of moderate-to-severe depressive symptoms), (4) a current major depressive episode of > 3 months, (5) either antidepressant naïve or had a wash-out period of 5 or more half-lives for psychotropic medications, and (6) fluency in English. Individuals who have failed four or more adequate pharmacological treatments for MDD or have previously failed to respond to ESC or ARI were excluded. A complete list of exclusion and inclusion criteria can be found in Lam et al. (2016).

Study design

The total study duration was sixteen weeks. During Phase I (weeks 0-8), participants were initiated on open-label ESC at a dosage of 10 mg/day and increased up to 20 mg/day based on the judgement of the physician effectiveness/tolerance. At week 8, participants attaining a > 50 % reduction in baseline MADRS score were classified as "week 8 responders" and continued to receive ESC, while participants who did not respond to ESC monotherapy at week 8 were classified as "week 8 non-responders" and received open-label adjunctive ARI (2–10 mg/d, flexible-dosage) while continuing ESC during Phase II (weeks 8-16).

Study measures

Depressive symptoms were assessed biweekly using MADRS from weeks 0 to 16. Treatment outcomes were: (1) response status on the last visit of Phases I and II with "responders" demonstrating a reduction of ≥ 50 % in MADRS from baseline, (2) remitter status at the end of Phases I and II with participants achieving a MADRS total score of 10 or less classified as "remitters", and (3) the percentage of symptom improvement across visits during Phases I and II calculated by subtracting the total MADRS score from the baseline score and then dividing by the baseline score for each of the eight timepoints.

The Toronto Side Effects Scale (TSES), administered on weeks 2, 4, 10, 12, and 16, is an evaluation of the frequency and severity of treatment-related side effects on a 5-point Likert scale grouped into four categories: central nervous system, gastrointestinal tract, sexual function, and weight gain. The side effect intensity score was calculated by multiplying the frequency and severity of each side effect. The measures of treatment-related side effects were: (1) the presence or absence of each category of side effect at the end of Phases I and II, and (2) the intensity of each category of side effect across visits during each Phase.

Serum concentrations of ESC, ARI, and their respective major metabolites, S-desmethylcitalopram (S-DCT) and dehydroaripiprazole (DHA), were available for weeks 2, 10, and 16 for ESC and at weeks 10 and 16 for ARI. Concentrations were measured by a validated liquid chromatography-mass spectrometry method described in the Supplementary Material.

DNA genotyping data

SNPs and haplotypes for *CYP2C19*, *CYP2D6,* and *CYP3A4* that are associated with altered metabolism and are common in the reference population (consisting of Europeans, African Americans, and East Asian ancestry) were included for genotyping. *ABCB1* SNPs were chosen based on previously reported associations between P-gp substrates and treatment outcomes [9]. Genomic DNA was isolated from blood samples using a modified FlexiGene DNA kit (QIAGEN, Hilden, Germany). Genotyping was performed using standard TaqMan assays (Thermo Fisher Scientific, ON, Canada) for nine *CYP2D6* SNPs (rs1065852, rs1135840, rs16947, rs28371706, rs28371725, rs35742686, rs3892097, rs5030655, rs5030656), three *CYP2C19* SNPs (rs12248560, rs4244285, and rs4986893), two *CYP3A4* SNPs (rs11773597 and rs28371759), and six *ABCB1* SNPs (rs1045642, rs1128503, rs2032582, rs2032583, rs2235015, and rs2235040). TaqMan *CYP2D6* copy number variations were assessed using copy-number assays (Thermo Fisher Scientific) and CopyCaller Version 2.1 (Applied Biosystems, Burlington, ON, Canada). TaqMan genotyping assay IDs for *CYP2C19* and *CYP2D6* are listed in **Table S1**. Quality control procedures for genotyping are described in detail in Supplementary Material.

DNA methylation data

Blood samples for DNAm analysis were collected at baseline prior to the start of treatment. Since the current study is a secondary hypothesis-driven analysis, we extracted post-quality control (QC) DNAm levels for our genes of interest, *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1*, from our previous genome-wide DNAm analysis. This genome-wide DNAm analysis, QC, and pre-processing were conducted at the McGill University and Genome Quebec Innovation Center (GQIC) (Montreal, Canada), which is described in detail elsewhere [28].

Briefly, genome-wide DNAm was quantified using the Infinium MethylationEPIC Beadchip (Illumina, USA) following the extraction of DNA from whole blood samples. DNAm sample QC, pre-processing, and analysis were performed using the Chip Analysis Methylation Pipeline (ChAMP) Bioconductor package in R 3.4 [29] after accounting for attrition rates. Probes with low signal detection relative to control probes that have less than three beads in > 5 % of samples, cross-reactive probes, non-CpG probes, sex chromosome probes, and probes that hybridize to SNPs were removed.

DNAm levels were expressed as beta-values for each CpG site, which is a ratio of methylated signal to the sum of unmethylated and methylated signal, adjusted for batch. Post-QC, we were left with a total of 60 CpG sites across our four genes of interest to be included in our analyses (**Table S2**). CpG islands (CGI) in TSS200, TSS1500, 5′UTR, 3′UTR, 1st exon, gene body, exon boundaries, and enhancers for each gene were obtained using the methylation probe annotation file from Illumina and extracted using GenCode Basic v12, ENCODE, RefSeq, and FANTOM5.

Gene expression data

Blood samples for mRNA analysis were collected at weeks 0, 2, and 8. mRNA sequencing procedures and pre-processing were performed at McGill University and GQIC as described elsewhere [28, 30]. Total RNA was extracted from whole blood samples using a modified version of the LeukoLOCK Total RNA Isolation System

protocol and genomic DNA was removed using DNase treatment. Libraries were prepared using Illumina TruSeq mRNA stranded protocol following the manufacturer's instructions. Only samples with RNA Integrity Number (RIN) ≥ 6.0 were used and sequenced by the Illumina HiSeq4000 with 100 nt paired-end reads. FASTXToolkit was used for preprocessing and Trimmomatic for adapter trimming. Tophat2 was used to align the reads to the reference genome and reads that lost their mates through the cleaning process aligned independently from reads that still had their pairs. Gene expression was quantified using HTSeqcount and a reference transcript annotation from ENSEMBL. Normalization was performed using DESeq2.

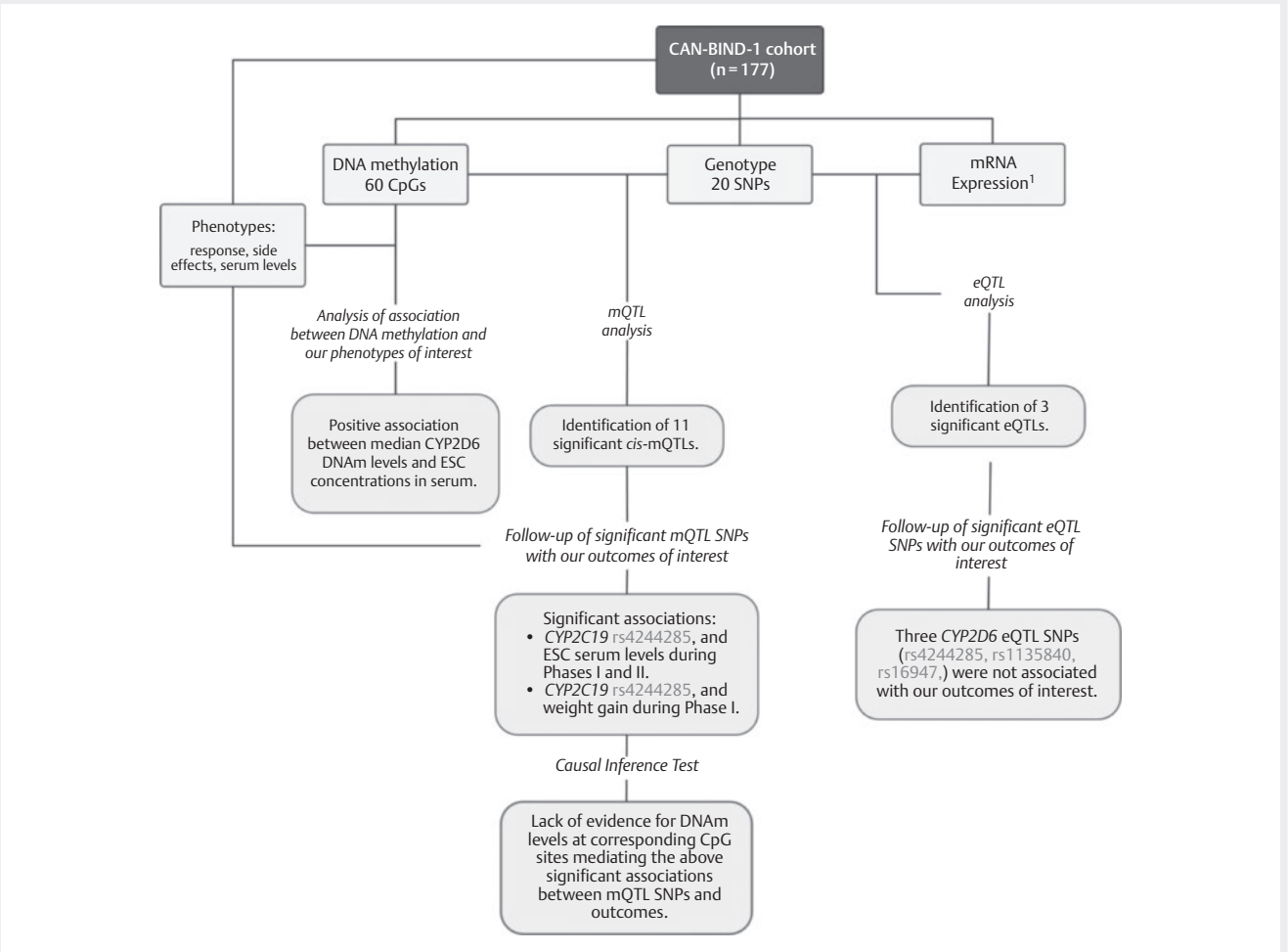
Statistical analyses

All analyses were conducted using R Version 4.3.2 (R Foundation for Statistical Computing Platform, 2022) and Rstudio Version 2023.12.1 (Rstudio Inc, 2023). Phases I and II were analyzed separately. Furthermore, given the different metabolic pathways of ESC and ARI, ESC-Only (i.e., week 8 responders) and ESC + ARI (i.e., week 8 non-responders) treatment arms were analyzed separately for Phase II.

A false discovery rate (FDR) approach was used to control for multiple comparisons in the analysis of each subsample (i.e., total sample for Phase I and by treatment arms for Phase II) with a significance threshold of $q < 0.05$ (two-tailed). For *post-hoc* and exploratory analyses, $p < 0.05$ was considered statistically significant. ▶ **Fig. 1** is an overview of the study design and workflow. **Figure S1** provides a workflow of the statistical tests conducted for the association analyses.

Association of baseline DNA methylation with clinical and pharmacokinetic outcomes

Our targeted study covered 60 CpG sites across our four pharmacogenetic genes of interest (*CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1*). Baseline DNAm levels at the 60 CpG sites and median DNAm levels at each gene were included as an independent vari-



▶ **Fig. 1** Analysis flowchart of the study and results for identifying epigenetically mediated associations between *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1*single nucleotide polymorphisms and treatment response, side effects, and pharmacokinetic outcomes. Green boxes indicate results. [1]Gene expression data was available only for *CYP2D6* and *ABCB1* and for three time points dext-linkng Phase I: weeks 0, 2, and 8. Note: DNAm = DNA methylation; eQTLs = Expression quantitative trait loci, the association between SNPs and mRNA expression levels; ESC = Escitalopram; mQTLs = Methylation quantitative trait loci, the association between SNPs and methylation level; S-DCT = S-desmethylcitalopram; SNPs = single nucleotide polymorphisms.

able in the following analyses [31, 32]. Multiple logistic regression models were conducted to evaluate the association between beta-values at *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1* CpG sites and our categorical outcomes for response and remission (responder vs. non-responder and remitter vs. non-remitter) and side effects (present vs. absent) at weeks 8 and 16. Given the availability of longitudinal data for MADRS and TSES scores, symptom improvement and side effects (intensity of each category of side effect) were assessed using linear mixed-effects models that included an interaction between DNAm level at each CpG site or median DNAm level for each gene with timepoint. Finally, linear regression models were conducted to evaluate the association between DNAm and our pharmacokinetic outcomes: serum levels of ESC and ARI, their metabolites, and metabolite-to-drug ratio. These analyses were all adjusted for age, sex, ancestry, and recruitment site. The latter was included as a random effect factor along with subject ID in the linear mixed-effects models.

Methylation quantitative trait loci analysis

We used linear regression models to identify mQTLs: DNAm level at each of the 60 CpG sites was the dependent variable and genotypes for each of the 20 SNPs (encoded as 0, 1, or 2 according to the number of minor alleles) were included as an independent variable. The models were adjusted for age, sex, and ancestry, which have been shown to be associated with DNAm variability [33, 34]. A total of 1,200 SNP-CpG pairs were tested and FDR was used to correct for multiple testing ($q < 0.05$).

To distinguish between local (*cis*) and distant (*trans*) mQTLs, *cis*-mQTLs were defined as the associated SNP being less than 500 kb upstream and downstream from the CpG site. This study focused on the *cis*-effects [18]; therefore, only identified *cis*-mQTLs with an association of $q < 0.05$ adjusted for multiple testing were included in the downstream analysis.

Next, we investigated the effect of *cis*-mQTL SNPs identified in the steps above on treatment response and remission, side effects, and drug serum levels using association tests described in **Figure S1**. For significant associations, to determine whether DNAm levels at the corresponding CpG site of the mQTL SNP were interacting with or mediating the effect of the SNP on the phenotype, we performed likelihood ratio tests (LRT) and causal inference tests (CITs), respectively. LRTs compared models with and without an interaction between the SNP-CpG pair on their goodness of fit with a $p < 0.05$, indicating a significant effect of the SNP x CpG interaction on the phenotype. CITs were performed using the "cit" package in R to identify mQTL SNPs whose association with our clinical and pharmacokinetic outcomes was significantly mediated by DNAm at the corresponding CpG site ($p < 0.05$).

CIT is used to assess the relationship between a causal variable (genotype, G), a potential mediator (DNAm, M), and a phenotypic trait (our outcomes of interest, P) [35, 36]. The steps are illustrated in **Figure S2**. To clarify that DNAm acts as a mediator of the effect of the mQTL SNP on our outcomes, the following criteria must be simultaneously true: 1) G and P are associated, 2) G and M are associated after adjusting for P, 3) M and P are associated after adjusting for G, and 4) P is independent of G after adjusting for M. If DNAm is independently influenced by G rather than being a mediator of the effect of G on P, the estimated effect of G on P should not be impacted when conditioning on M. However, if DNAm is indeed a mediator, conditioning on M should considerably reduce the observed effect of G on Y. The covariates, age, ancestry, and sex were adjusted in the above four tests of causality. Given that this is a *post-hoc* analysis, the significant threshold for the CITs was set at $p < 0.05$.

Expression quantitative trait loci (eQTL) analysis

Gene expression data was detectable for *CYP2D6* and *ABCB1* in blood. Gene expression data for *CYP3A4* and *CYP2C19* was under the threshold for detection in whole blood and could not be accurately quantified. To elucidate the functional mechanisms by which *cis*-mQTL SNPs and CpG sites of *CYP2D6* and *ABCB1* influence our study outcomes, we determined the effects of SNP and DNAm on mRNA expression levels, using eQTL analyses for the SNP and mRNA association pair. Linear regression analyses were conducted with log2 transformed mRNA expression level as the dependent variable and *cis*-mQTL SNP as independent variables. Age, sex, and ancestry were included as independent covariates. FDR was used to correct for multiple testing ($q < 0.05$). CITs were performed to identify the DNAm-mediated association between eQTL SNPs and mRNA levels.

Results

Participant characteristics

Two hundred and eleven participants were recruited from six clinical research centers across Canada [10, 25]. There were 31 dropouts by week 8 who lacked MADRS scores and drug serum levels for Phases I and II, thus were not included in the current analyses. Three individuals lacked sufficient genetic material for genotyping and DNAm analysis. Therefore, analysis was conducted on a total of 177 participants. ▶ **Table 1** summarizes the sample characteristics.

The majority of the participants were female (63 %) and of European ancestry (72 %). By week 8, 47 % were responders and continued ESC (n = 83). The week 8 non-responders (n = 94) were augmented with ARI for the remainder of the trial. At week 16, responders made up 91 % and 60 % of those in the ESC-Only group and the ESC + ARI treatment group, respectively.

For *CYP2D6*, the effect of genotype on enzymatic function was unclear for five participants and genotyping was consistently unsuccessful for one participant. For *CYP2C19,* there was one participant with a poor-quality sample. Therefore, analyses pertaining to *CYP2C19* and *CYP2D6* SNPs were conducted on 177 participants for *CYP2C19* and 172 participants for *CYP2D6*.

Single CpG site and median DNA methylation level analysis

Associations between DNAm levels of individual CpG sites of the *CYP2C19, CYP2D6, CYP3A4,* and *ABCB1* genes in whole blood and measures of treatment response, remission, and side effects were not significantly associated following FDR corrections.

For Phase I, linear regressions revealed that the median baseline DNAm level of *CYP2D6* was significantly associated with dose-adjusted week 2 serum concentrations of ESC (N = 163, F(1,152) =

▶ **Table 1** Basic sample demographics and clinical information by treatment arm.

	All	Phase II Treatment Arm		
Variables	**N = 177**	**ESC-Only (N = 83)**	**ESC + ARI (N = 94)**	***p*-value[1]**
Age	35.43 (12.70)	34.64 (12.33)	36.13 (13.05)	0.488
Sex				0.358
Female	111 (63 %)	55 (66 %)	56 (60 %)	
Male	66 (37 %)	28 (34 %)	38 (40 %)	
Ancestry				0.994
Non-European [African (4), East Asian (14), Latin American (9), South Asian (5), Southeast Asian (4), and mixed ancestry (13)]	49 (28 %)	23 (28 %)	26 (28 %)	
European	128 (72 %)	60 (72 %)	68 (72 %)	
Previous antidepressant treatment for current MDE				**0.009**
None	101 (57 %)	56 (67 %)	45 (48 %)	
1 +	76 (43 %)	27 (33 %)	49 (52 %)	
ESC Dose at week 8				> 0.999
10 mg	176 (99 %)	83 (100 %)	93 (99 %)	
20 mg	1 (0.6 %)	0 (0 %)	1 (1.1 %)	
ESC Dose at week 16				0.487
10 mg	16 (9.9 %)	10 (13 %)	6 (7.0 %)	
15 mg	2 (1.2 %)	1 (1.3 %)	1 (1.2 %)	
20 mg	144 (89 %)	65 (86 %)	79 (92 %)	
Baseline MADRS	29.95 (5.48)	29.10 (5.45)	30.71 (5.42)	**0.027**
%Δ in MADRS from baseline				
Week 8	45.95 (31.97)	73.21 (16.08)	21.88 (21.37)	**< 0.001**
Week 16	65.20 (27.18)	77.99 (20.81)	54.14 (27.28)	**< 0.001**
Response Status at week 16				**< 0.001**
Responder	121 (74 %)	69 (91 %)	52 (60 %)	
Non-Responder	42 (26 %)	7 (9.2 %)	35 (40 %)	
Remission Status at week 16				**< 0.001**
Remitter	97 (60 %)	61 (80 %)	36 (41 %)	
Non-Remitter	66 (40 %)	15 (20 %)	51 (59 %)	

[1]P-values were derived from: Wilcoxon rank sum test (continuous variables); Pearson's Chi-squared test (categorical variables); Fisher's exact test (categorical measurement if Chi-squared assumptions were not met). ESC, escitalopram; ARI, aripiprazole; MDE, major depressive episodes; MADRS, Montgomery-Åsberg Depression Rating Scale.

13.19, $p < 0.001$, $q = 0.019$), explaining 18.5 % of the variance in serum levels (**Figure S3**). Higher ESC_{adj} concentration in serum was observed at week 2 with increasing levels of median DNAm of the *CYP2D6* gene. DNAm levels at individual CpG sites of *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1* genes were not significantly associated with serum drug and metabolite concentrations following FDR corrections.

Methylation quantitative trait loci analyses

Identification of methylation quantitative trait loci

We performed linear regression analyses including the entire sample (n = 177) to reveal associations between genotypes of the 20 SNPs and the methylation level of the 60 CpG sites across the *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1* genes. Among the 1200 tested SNP-CpG pairs using linear regression tests, a total of 11 pairs were statistically significant following multiple testing corrections ($q < 0.05$), which are shown in ▶ **Table 2**. Since the distance between all SNP-CpG pairs was less than 500 kb, all the identified mQTLs were in *cis*. These 11 SNP-CpG pairs were composed of four unique SNPs (rs4244285 in *CYP2C19,* and rs1065852, rs1135840, and rs16947 in *CYP2D6*) and eight CpG sites (cg00051662, cg02808805, and cg20031717 in *CYP2C19*, and cg15597984, cg24307449, cg09322432, cg04692870, and cg17498424 in *CYP2D6*). The density distributions by genotype for *cis*-mQTLs are shown in ▶ **Fig. 2**.

For the three *CYP2D6* mQTL SNPs, rs1065852, rs1135840, and rs16947, the r^2 ranged between 0.13-0.44 and the D' was 1 in Europeans using the "LDlinkR" R package. Thus, these SNPs were analyzed separately as the extent of LD was not clear.

These analyses were repeated in the European subsample to account for heterogeneity in ancestry within the sample. Here, a total of four significant CpG-SNP pairs were identified ($q < 0.05$), all of which also overlapped with the ones identified in the whole sample (**Figure S4**).

▲ **Table 2** Methylation quantitative trait locus (mQTL) analysis between single nucleotide polymorphisms (SNPs) and CpG sites.

Gene	SNP	Variant	CpG Site	CpG Genomic Location	*Europeans*			*All participants*			
					F-Stat[1]	R^2	*p*-value	*q*-value	F-Stat[2]	R^2	*p*-value	*q*-value
CYP2C19	rs4244285	681 G>A	cg00051662	TSS1500	5.45	0.08	5.45×10^{-3}	0.274	9.67	0.10	1.06×10^{-4}	**1.15×10^{-2}**
CYP2C19	rs4244285	681 G>A	cg02808805	TSS1500	15.52	0.21	1.04×10^{-6}	**5.60×10^{-4}**	17.54	0.17	1.23×10^{-7}	**1.48×10^{-4}**
CYP2C19	rs4244285	681 G>A	cg20031717	Body	11.80	0.16	2.13×10^{-5}	**7.66×10^{-3}**	12.01	0.13	1.34×10^{-5}	**2.69×10^{-3}**
CYP2D6	rs1065852	100 C>T	cg15597984	TSS200	7.79	0.09	6.65×10^{-4}	0.111	11.61	0.21	2.72×10^{-7}	**1.63×10^{-4}**
CYP2D6	rs1065852	100 C>T	cg24307449	TSS200	7.98	0.09	5.61×10^{-4}	0.111	10.42	0.11	5.35×10^{-5}	**7.13×10^{-3}**
CYP2D6	rs1065852	100 C>T	cg09322432	TSS1500	3.19	0.02	4.47×10^{-2}	0.640	11.73	0.11	1.74×10^{-5}	**2.99×10^{-3}**
CYP2D6	rs1135840	4180 G>C	cg04692870	Body	15.77	0.20	8.55×10^{-7}	**5.60×10^{-4}**	15.74	0.17	5.49×10^{-7}	**2.20×10^{-4}**
CYP2D6	rs1135840	4180 G>C	cg15597984	TSS200	5.72	0.06	4.27×10^{-3}	0.271	11.32	0.17	2.47×10^{-5}	**3.70×10^{-3}**
CYP2D6	rs1135840	4180 G>C	cg24307449	TSS200	6.72	0.07	1.72×10^{-3}	0.169	13.02	0.13	5.58×10^{-6}	**1.34×10^{-3}**
CYP2D6	rs1135840	4180 G>C	cg17498424	TSS1500	11.01	0.13	4.12×10^{-5}	**1.11×10^{-2}**	10.30	0.10	6.05×10^{-5}	**7.26×10^{-3}**
CYP2D6	rs16947	2850 C>T	cg04692870	Body	6.94	0.10	1.41×10^{-3}	0.152	14.77	0.16	1.24×10^{-6}	**3.73×10^{-4}**

[1]Degrees of freedom for the F-statistic was (2, 118). [2]Degrees of freedom for the F-statistic was (2, 166). **Bold** indicates $q<0.05$. *Note:* Body = Between the ATG and stop codon, irrespective of the presence of introns, exons, transcriptional start site (TSS), or promoters; n.s. = Not significant; TSS200 = 200 bases upstream of the TSS; TSS1500 = 200–1500 bases upstream of the TSS.

Association of identified methylation quantitative trait loci in whole blood with clinical outcomes

We evaluated if the identified mQTL SNPs affected our response, remission, and side effects outcomes. Following FDR corrections, one mQTL SNP, *CYP2C19* rs4244285, was associated with the intensity of treatment-related weight gain between weeks 2 to 4 (n = 173, $F(2,167) = 9.96$, $p = 8.19\times10^{-5}$, $q = 0.027$) (▶ **Table 3**). *CYP2C19* rs4244285 genotypes, *1/*1, *1/*2, and *2/*2, can be classified phenotypically as normal metabolizers (NMs), intermediate metabolizers (IMs), and poor metabolizers (PMs), respectively [37]. 6.7 % of *1/*1 NMs (n = 119) and 14.9 % of *1/*2 IMs (n = 47) reported ≥ 2 lbs of treatment-related weight gain between weeks 2 to 4, whereas all of the *2/*2 PMs (n = 3) reported experiencing no treatment-related weight gain during this time frame (▶ **Fig. 3a-b** and for effect sizes, see **Table S3**). In terms of severity of the treatment-related weight gain (i.e., the extent to which the participant was troubled by the weight gain), 3.4 % of *1/*1 NMs and 6.3 % of *1/*2 IMs reported it to be ≥ 2 on a 5-point Likert scale.

Association of identified methylation quantitative trait loci in whole blood with serum drug concentrations

One mQTL SNP, *CYP2C19* rs4244285, was associated with dose-adjusted serum ESC concentrations at weeks 2, 10, and 16 (▶ **Table 3**). Concentrations of ESC_{adj} were approximately 120-200 % higher in the *2/*2 PMs (n = 3) and 38-55 % higher in the *1/*2 IMs (n = 50) compared to those with the *1/*1 NMs (n = 120) (▶ **Fig. 3b-c**, see **Table S3** for effect sizes).

DNA methylation-mediated methylation quantitative trait loci associations

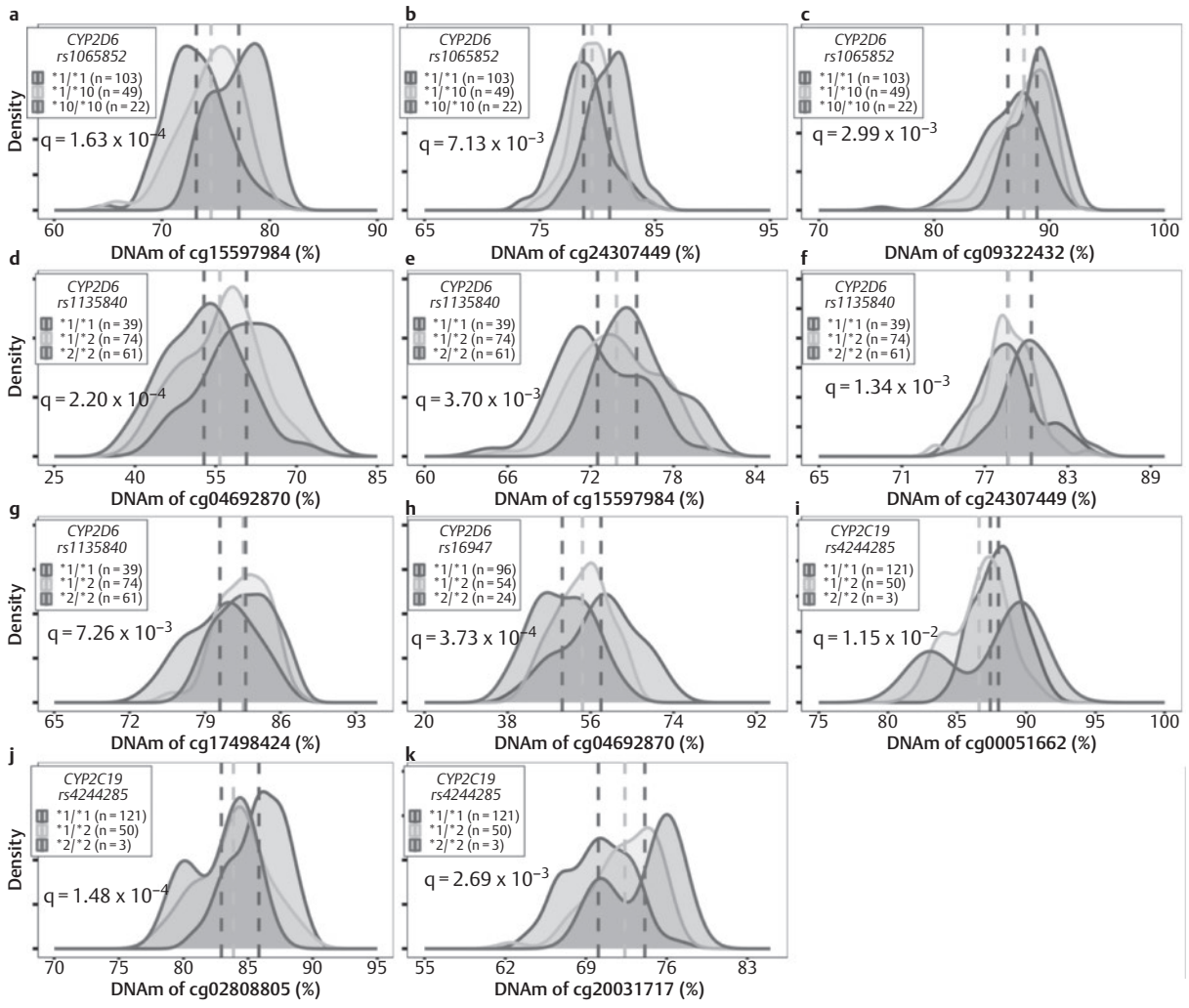
CITs revealed that significant associations between mQTL SNP, *CYP2C19* rs4244285, and treatment-related weight gain and concentrations of ESC_{adj} were not being mediated by DNAm levels at the three corresponding CpG sites, cg00051662, cg02808805, and cg20031717. LRTs comparing regression models, including a SNP x CpG interaction against the model without the CpG site, did not indicate a significant interaction effect between SNP and DNAm on these outcomes.

Expression quantitative trait loci analysis

Identifying expression quantitative trait loci

mRNA levels were detectable only for *CYP2D6* and *ABCB1* in whole blood at weeks 0, 2, and 8. For *CYP2D6*, three eQTL SNPs, rs1065852, rs1135840, and rs16947, were identified and survived FDR corrections (**Table S4**).

For *CYP2D6* rs1065852, the *10/*10 (i.e., PMs) and *1/*10 (i.e., IMs) groups had, on average, approximately 9 % and 7 % higher mRNA expression than the *1/*1 (i.e., NMs) group across time points (▶ **Fig. 4a**). For *CYP2D6* rs1065852 and rs16947, all three genotype groups are classified phenotypically as NMs. With an increasing number of the *2 alleles, more gene expression was observed for both SNPs (▶ **Fig. 4b-c**). *CYP2D6* rs1065852 *2/*2 and *1/*2 groups on average had 17 % and 10 % higher gene expression than the *1/*1 group. Likewise, *CYP2D6* rs16947 *2/*2 and *1/*2 groups, on average, had 6 % and 4 % higher gene expression than those with the *1/*1 genotype. CITs revealed that DNAm lev-

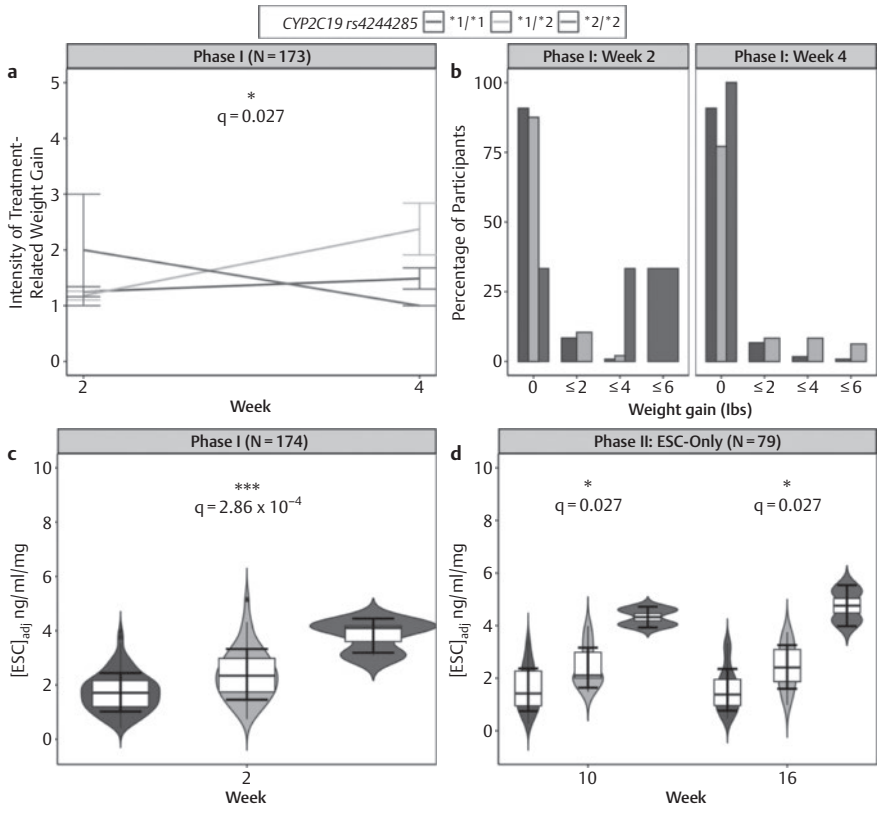


▶ **Fig. 2** Density plots of *cis*-mQTLs identified in the CAN-BIND-1 sample. a-k. Significant associations between SNP and DNAm of CpG sites in whole blood, adjusted for age, ancestry, and sex. The methylation density plot is color-coded by genotype. The number of individuals in each genotype group is shown in the top left corner. The dotted line indicates the mean. *Note*: DNAm = DNA methylation; mQTL = methylation quantitative trait loci; SNP = single nucleotide polymorphisms.

▶ **Table 3** Analyses of association between identified mQTLs with outcomes of interest.

Gene	mQTL SNP	Genotypes (n)	Phenotype	F-Stat	*df*	*p*-value (G vs P)	*q*-value (G vs P)
Phase I: All							
CYP2C19	rs4244285	*1*/1 (n = 120), *1/*2 (n = 20), *2/*2 (n = 3)	Weight gain from weeks 2-4	9.96	2, 167	8.19×10^{-5}	0.027
CYP2C19	rs4244285	*1*/1 (n = 120), *1/*2 (n = 20), *2/*2 (n = 3)	Week 2 ESC_{adj} concentrations	16.65	2, 152	2.87×10^{-7}	2.87×10^{-4}
Phase II: ESC-Only							
CYP2C19	rs4244285	*1*/1 (n = 55), *1/*2 (n = 22), *2/*2 (n = 2)	Week 10 ESC_{adj} concentrations	12.09	2, 55	4.43×10^{-5}	0.027
CYP2C19	rs4244285	*1*/1 (n = 55), *1/*2 (n = 22), *2/*2 (n = 2)	Week 16 ESC_{adj} concentrations	12.51	2, 55	3.32×10^{-5}	0.027

Note: ESC = escitalopram; G = genotype; mQTL = methylation quantitative trait locus; P = phenotype.



▶ **Fig. 3** Significant mQTL SNPs associated with clinical and pharmacokinetic outcomes. One significant mQTL SNP, *CYP2C19* rs4244285, was associated with our outcomes of interest: (**a**-**b**) *CYP2C19* rs4244285 was associated with treatment-related weight gain between weeks 2 to 4 dext-linkng Phase I with 6.7 % and 14.9 % of participants with the *1/*1 (n = 119) and *1/*2 (n = 47) genotypes, respectively, reporting ≥ 2 lbs of treatment-related weight gain, whereas none of those with the *2/*2 genotype (n = 3) reported weight gain. (**b**-**c**) Relative to those with the *1/*1 genotype, those with the *1/*2 and *2/*2 genotypes of *CYP2C19* rs4244285 showed higher ESC_{adj} serum concentrations at weeks 2, 10, and 16 dext-linkng Phase I and in ESC-Only dext-linkng Phase II adjusted for age, ancestry, recruitment site, sex, and time since last ESC dose. Specifically, the *2/*2 group demonstrated the highest ESC_{adj} concentrations with those having *1/*2 had concentrations between the two groups. *Note*: ARI = Aripiprazole; ESC = Escitalopram; mQTLs = Methylation quantitative trait loci, the association between SNPs and methylation level; S-DCT = S-desmethylcitalopram. ***q < 0.001, **q < 0.01, *q < 0.05

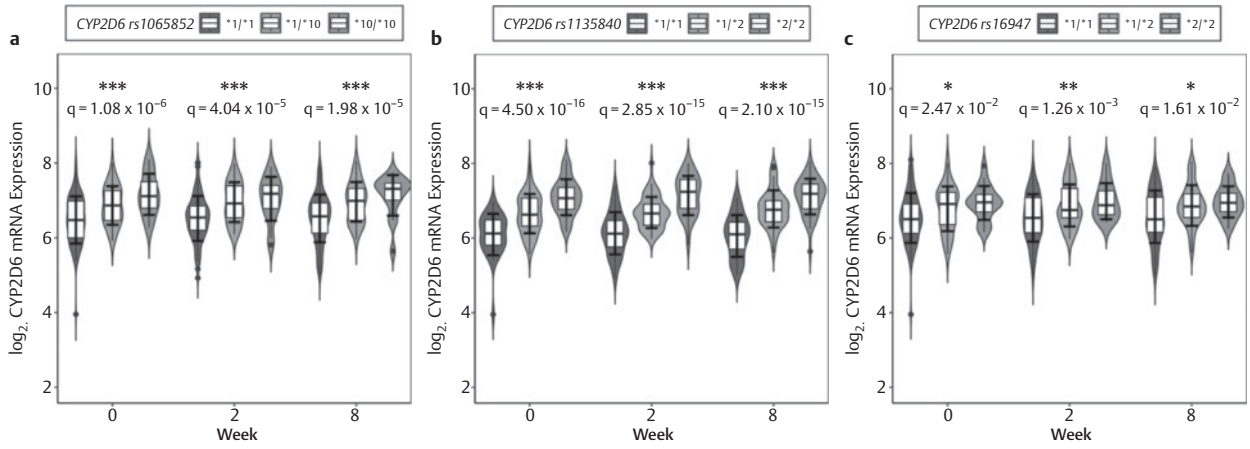
els at corresponding CpG sites were likely not mediating the effect of the eQTL SNPs on mRNA levels.

The *CYP2D6* eQTL SNPs, rs1065852, rs1135840, and rs16947, were not significantly associated with measures of response, side effects, or drug serum concentrations.

Discussion

We and others have shown that polymorphisms in pharmacokinetic genes *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1* can contribute to various degrees of variations in serum concentrations, response, and certain side effects to the antidepressant ESC [9, 10]. Recent research has also shown that genetic variants can impact patterns of DNAm and gene expression dynamics [38, 39]. Based on these observations, we conducted integrative multi-omic analyses of genotypic, methylomic, and transcriptomic data to explore their combined contributions to ESC treatment response, side effect profile, and serum concentrations.

In this study, we showed that DNAm at individual CpG sites of *CYP2C19*, *CYP2D6*, *CYP3A4*, and *ABCB1* were not associated with measures of response, side effects, and drug serum levels. Instead, we observed that a higher median DNAm level of *CYP2D6* was associated with higher week 2 serum levels of ESC_{adj}. In our QTL analyses, we identified eleven *cis* SNP-CpG pairs with test statistics exceeding the 5 % FDR threshold, which constituted four unique mQTL SNPs and eight unique CpGs. Sequence variation in these mQTL SNPs may influence DNAm levels at the corresponding CpG sites. Of the four mQTL SNPs, there was one SNP, *CYP2C19* rs4244285, that showed associations with specific outcomes of interest, indicating that it may contribute to these outcomes via effects on local DNAm. Specifically, *CYP2C19* rs4244285 was associated with concentrations of ESC_{adj} at each timepoint, as well as treatment-related weight gain during Phase I. When we conducted follow-up CITs, the results did not provide compelling evidence that DNAm might have a mediating role in the effects at these loci. We also showed that *CYP2D6* rs1065852, rs1135840, and rs16947



▶ **Fig. 4** Significant eQTLs for *CYP2D6*. Identified eQTLs in whole blood for *CYP2D6* at weeks 0, 2, and 8: (**a**) rs1065852, (**b**) rs1135840, and (**c**) rs16947. *Note*: eQTLs = Expression quantitative trait loci, the association between SNPs and mRNA expression levels. ***q<0.001, **q<0.01, *q<0.05.

were eQTLs, which were not associated with our outcomes of interest.

The *CYP2C19* mQTL SNP, rs4244285, is a functional polymorphism that captures the *1 and *2 alleles. Although we did not observe an association between *CYP2C19* rs4244285 and response/remission following treatment with ESC, previous studies have shown mixed results. Some studies showed less symptom improvement in *2/*2 PMs relative to *1/*1 NMs [40, 41], while others showed the opposite effect [42, 43].

Here, we showed an association between *CYP2C19* rs4244285 and early weight gain during treatment, with *1/*2 IMs experiencing the most weight gain between weeks 2 to 4 followed by *1/*1 NMs, while *2/*2 PMs did not report any treatment-related weight gain. Interestingly, two previous studies showed an association between treatment-related weight gain and *CYP2C19* metabolizer phenotype with IMs and PMs of *CYP2C19* associated with more weight gain after 45 days of treatment with ESC or citalopram [40] and also after six months on citalopram [44]. The discrepancy between these and our findings may be due to the low number of PMs (n = 3) we had in our sample, resulting in being underpowered to detect an association. Another notable difference between these findings may be due to the duration of ESC/citalopram treatment. Weight loss was observed in PMs in the shorter term (e.g., two weeks) in our study, while weight gain was observed in IMs and PMs with longer-term treatment in the two previous studies. Acute serotonin reuptake inhibition has been reported to help suppress appetite and/or hunger sensations and increase satiety, whereas prolonged serotonin reuptake inhibition produces increased extracellular synaptic serotonin concentrations, resulting in the blockade of serotonin receptors. This prolonged serotonin reuptake inhibition subsequently causes cravings for carbohydrate-rich foods and ultimately may result in SSRI-related weight gain in the long term [45]. It is unclear whether with long-term use of ESC, we would observe a different trend between *CYP2C19* rs4244285 and weight gain in our sample.

Typically, higher methylation of CpGs in gene promoter regions is associated with transcriptional repression, while in gene bodies, it is positively associated with gene expression [46]. *CYP2C19* rs4244285 *1/*2 IMs and *2/*2 PMs had lower methylation levels at the two TSS1500 promoter CpGs and higher methylation levels at the CpG located in the body relative to the *1/*1 NMs. This pattern of DNAm would suggest that those with rs4244285 *1/*2 and the *2/*2 genotypes, or IM and PMs, may have higher *CYP2C19* gene expression. However, since *CYP2C19* gene expression levels were below the detection level in whole blood to carry out eQTL analysis, the functional impacts that this SNP and differential methylation within these CpGs have on *CYP2C19* expression levels is unclear and need to be further explored.

Besides DNAm, various other post-transcriptional modifications that can also significantly alter gene expression and drug effects, including microRNAs (miRNAs). *CYP2C19* rs4244285 has also been reported to be a mirSNP, which may regulate *CYP2C19* gene expression by interfering with normal miRNA function [47]. One study reported that levels of specific miRNAs, hsa-miR-1343-3p, and hsa-miR-6783-3p, were significantly upregulated in individuals with the *1/*2 and *2/*2 genotype, and these miRNAs down-regulated the protein expression of *CYP2C19*[47]. These results align with current and previous observations of higher unmetabolized parent drug concentrations in those with the *1/*2 and *2/*2 genotypes relative to the *1/*1 genotype for whom the CYP2C19 protein is more active [41, 43].

Despite the novel and promising results detailed here, our targeted study has several important limitations. (1) Since our analysis was exploratory, no priori power analysis was conducted. The relatively small sample size may offer limited statistical power in detecting minor or modest effect mQTLs. (2) Specific to epigenetic analyses, we designed a hypothesis-driven study focused on four

pharmacokinetic genes of interest in antidepressant drug metabolism. While we did not examine genetic variation or DNAm across the entire genome, SNPs and haplotypes selected for *CYP2C19*, *CYP2D6,* and *CYP3A4* cover > 95 % of the common alleles associated with altered metabolism. Thus, we studied only these select SNPs, which will not fully capture the total variation in these genes. (3) We also recognize the importance of including environmental and lifestyle factors that are known to influence epigenomic modifications, such as smoking status, cannabis use, alcohol consumption, diet, and stress, where possible. In addition, cytosine modification has been shown to be influenced by circadian rhythm [48]. However, as time-of-day or other environmental factors were not collected as part of the CAN-BIND-1 study, these factors could not be included in our analyses. (4) Tissue and cell-specificity are known to significantly impact DNAm patterns and can lead to spurious associations if not appropriately addressed. In our current sample, no significant differences were observed in blood cell count (i.e., lymphocytes, monocytes, neutrophils, eosinophils, and basophils) comparisons between non-responders and responders (Ju et al., 2019). As DNA sample collection, as well as quality control and preprocessing of the Illumina EPIC array data, occurred previous to our study, and methods were used to extract a selection of 60 CpG probes within our four genes of interest, we were unable to use cell sorting techniques or to employ computational methods to correct for cellular heterogeneity. Similar approaches using this data have been published recently [32]. Although biomarker discovery that focuses on peripheral whole blood samples is far more feasible than using liver or brain samples, further work is required to understand how DNAm and gene expression differences in the blood might relate to the differences in these tissues of relevance [49, 50].

Genetic variations in pharmacokinetic genes have been studied extensively in pharmacogenetic research of antidepressant response and, therefore, are good candidates to study SNP-DNAm-gene expression relationships. Although we did not observe any associations between mQTLs of our targeted pharmacokinetic genes with antidepressant treatment response or remission, perhaps reflecting our relatively small sample, our results provide new insights into the regulation patterns among SNP, DNAm, and mRNA expression and also increase our understanding of functional mechanisms underlying established associations between pharmacokinetic genes and drug serum concentrations. Although these findings require to be validated in a larger independent sample, the identified *CYP2C19* mQTL SNP can serve as a starting point for further investigations on genetic biomarkers of antidepressant treatment-related weight gain.

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Conflict of Interest

Dr. Benicio N. Frey has no conflicts to disclose. Dr. Roumen V. Milev has received consulting and speaking honoraria from AbbVie, Allergan, Eisai, Janssen, KYE Pharmaceuticals, Lallemand, Lundbeck, Otsuka, and Sunovion, and research grants from CAN-BIND, Canadian Institutes of Health Research (CIHR), Janssen, Lallemand, Lundbeck, Nubiyota, Ontario Brain Institute (OBI), and Ontario Mental Health Foundation (OMHF). Dr. Sagar V. Parikh has received research support or consulting income from Aifred, Assurex (Myriad), Janssen, Mensante, Otsuka, Sage, and Takeda. Dr. Stefanie Hassel has no conflicts of interest to disclose. Dr. Pierre Blier received honoraria for participation in advisory boards, giving lectures, and/or expert consultation from Allergan, Bristol Myers Squibb, Janssen, Lundbeck, Otsuka, Pierre Fabre Medicaments, Pfizer and Sunovion; he received grants from Allergan, Janssen, and Lundbeck/Otsuka. Dr. Faranak Farzan received funding from Michael Smith Foundation for Health Research, Natural Sciences and Engineering Research Council of Canada Discovery, and Canadian Institutes of Health Research. Dr. Raymond W. Lam has received honoraria for ad hoc speaking or advising/consulting, or received research funds, from Abbvie, Asia-Pacific Economic Cooperation, BC Leading Edge Foundation, Boehringer-Ingelheim, Canadian Institutes of Health Research (CIHR), Canadian Network for Mood and Anxiety Treatments, Carnot, Healthy Minds Canada, Janssen, Lundbeck, Michael Smith Foundation for Health Research, MITACS, Neurotorium, Ontario Brain Institute (OBI), Otsuka, Pfizer, Unity Health, and VGH-UBCH Foundation. Dr. Gustavo Turecki has received an Investigator-initiated grant from Pfizer Canada, and honoraria from Bristol-Meyers Squibb Canada and Janssen Canada. Dr. Susan Rotzinger has received grant funding from the Ontario Brain Institute (OBI), and Canadian Institutes of Health Research (CIHR), and holds a patent Teneurin C-Terminal Associated Peptides (TCAP) and methods and uses thereof. Dr. Stefan Kloiber has received honorarium for past consultation from EmpowerPharm. Dr. Sidney H. Kennedy has received research funding or honoraria from the following sources: Abbott, Alkermes, Allergan, Bristol-Myers Squibb (BMS), Brain Canada, Canadian Institutes for Health Research (CIHR), Janssen, Lundbeck, Lundbeck Institute, Ontario Brain Institute (OBI), Ontario Research Fund (ORF), Otsuka, Pfizer, Servier, Sunovion and holds stock in Field Trip Health. Dr. Daniel J. Müller reports to be a co-investigator on two pharmacogenetic studies where genetic test kits were provided as in-kind contributions by Myriad Neuroscience. All other authors report no conflicts of interest related to this work.

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