An analysis of the effect of mu-opioid receptor gene (OPRM1) promoter region DNA methylation on the response of naltrexone treatment of alcohol dependence

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Received: 24 July 2019 / Revised: 22 January 2020 / Accepted: 27 January 2020 / Published online: 7 February 2020
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Abstract
This study explored the effect of OPRM1 promoter region DNA methylation on the outcome of treatment with the opioid antagonist naltrexone (NTX) for alcohol dependence (AD). Ninety-three patients with DSM-IV AD [41 African Americans (AAs) and 52 European Americans (EAs)] received double-blind treatment with NTX or placebo for at least three months. Relapse to heavy drinking was assessed during the first 13 weeks of the trial. Peripheral blood methylation levels of 33 CpG units in the OPRM1 promoter region were quantified using Sequenom EpiTYPER technology. Bayesian logistic regression was used to analyze the effects of NTX treatment, CpG methylation, CpG methylation × NTX treatment, and age on AD relapse. The Random Forest machine learning algorithm was applied to select AD relapse predictors. No significant effect of individual OPRM1 promoter CpG units on AD relapse was observed in either AAs or EAs. Age was significantly associated with AD relapse in EAs, among whom older subjects had a lower relapse rate. Random forest analyses revealed that the prediction rate for AD relapse reached 66.0% with five top variables (age and four CpG units; ranked by their importance to AD relapse) in the prediction model. These findings suggest that methylation levels of individual OPRM1 promoter CpG units do not contribute significantly to inter-individual variation in NTX response. However, the age of subjects in combination with a cluster of specific OPRM1 promoter CpG units may affect NTX treatment outcome. Additional studies of OPRM1 DNA methylation changes during and after NTX treatment of AD are needed.

Introduction
Naltrexone (NTX) and two other medications (disulfiram and acamprosate) have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of alcohol dependence (AD). NTX is an opioid antagonist, which blocks opioid receptors (particularly the mu-opioid receptor or MOR) and thus reduces the reinforcing effect of alcohol [1]. There is a high degree of variability in the response to NTX treatment among AD subjects. The efficacy of NTX treatment of AD depends on subjects’ genetic background [2], drinking situation (reward or relief drinking) [3], comorbid nicotine use or smoking status [4] and other factors.

Pharmacogenetic studies have been used to identify mechanisms underlying response variation in AD subjects receiving pharmacotherapy. Because NTX blocks opioid receptors, several clinical trials of the medication [5–9], including ours [10], have been conducted to examine the association between variation in opioid receptor genes and NTX treatment response. These studies focused on the potential moderating effect of a single nucleotide polymorphism (SNP rs1799971 or A118G) in Exon 1 of the MOR gene (OPRM1). The MOR is widely distributed throughout brain reward circuits, and it mediates the...
consumption and rewarding effects of alcohol and other substances including drugs of abuse [11, 12]. SNP rs1799971 results in a non-synonymous substitution of aspartate for asparagine in the amino terminus of the MOR, which has functional effects [13]. Although some of these studies showed differential responses to NTX as a function of the OPRM1 SNP rs1799971 [5, 7, 8], others found no significant effect of this variant on the outcome of NTX treatment [6, 9, 10]. The conflicting results suggest that other genetic factors or physiological or environmental factors (e.g., sex, age, diet, co-occurring diseases, and co-administered medications) may account for individual differences in response to NTX treatment.

The epigenetic state [e.g., DNA methylation (at CpG dinucleotides) and histone modifications (i.e., histone acetylation and methylation)] of genes changes during normal development and aging. Environmental factors can cause positive or negative epigenetic modifications with lasting effects on development, metabolism, and health [14], although an epigenetic state can be inherited meiotically as well as mitotically [15]. DNA methylation is the most widely studied epigenetic modification, which in mammals occurs mainly within the context of the CpG dinucleotide. Methylation of CpG sites (particularly those located in the promoter regions of genes) can either directly block transcription factor binding or attract methylated-CpG-binding proteins and other chromatin-remodeling enzymes to prevent the binding of transcription factors. Altered DNA methylation has been associated with a variety of diseases including AD [16]. Given the important role of the mu-opioid receptor in the reward pathway, several studies have examined DNA methylation patterns in the OPRM1 promoter region in subjects with alcohol or drug dependence. Increased OPRM1 promoter DNA methylation in heroin addicts has been reported [17, 18]. We observed hypermethylation of OPRM1 promoter CpG sites in AD subjects [19]. CpG sites in the promoter region of genes are usually unmethylated or hypomethylated [20]. Thus, an increase in methylation of OPRM1 promoter CpG sites may inhibit OPRM1 transcription (or MOR expression) as demonstrated in the study by Andria et al. [21], although OPRM1 promoter DNA methylation may not play a major role in regulating OPRM1 transcription [22]. However, the latter study only analyzed the correlation of the mean methylation level of 22 OPRM1 promoter CpGs and OPRM1 expression in postmortem brains of opiate addicts. In other words, it is unknown if the methylation of these 22 OPRM1 promoter CpGs can influence OPRM1 transcription individually or synergically. In addition, genetic variants such as the functional nonsynonymous variant (OPRM1 118A>G or rs1799971) can influence OPRM1 DNA methylation levels [23]. Because of the reduced availability of MORs, AD patients whose OPRM1 promoter CpG sites are hypermethylated may drink more alcohol to obtain the same euphoric effect as they did previously when they consumed less alcohol.

Because epigenetic variation modulates transcriptional networks and cellular functions, epigenetic markers are potential novel diagnostic tools for assessing disease phenotypes or predicting disease progression and treatment response. Pharmacoepigenetics, which studies the effect of epigenetic markers on variability and the underlying mechanism of drug response, is an emerging area of interest. There is also an increasing interest in developing therapeutic interventions that target epigenetic modifiers [such as DNA methyltransferases (DNMTs) and histone deacetyltransferases (HDACs)] for treating disease by reversing DNA and histone modifications [24]. Pharmacoepigenetics has been applied to identify epigenetic markers that predict the outcome of pharmacological treatments for cancers [25, 26], diabetes [27, 28], schizophrenia [29], depression [30], and Alzheimer’s disease [31]. For example, the DNA methylation state of the hyperpigmentation progressive 1 gene (HPP1) was identified as an early marker of response to combined therapy of metastatic colorectal cancer with fluoropyrimidine, oxaliplatin, and bevacizumab [26]. To date, no pharmacoepigenetic studies have examined the impact of epigenetic markers on the response to the pharmacotherapy of AD or other substance use disorders.

Our hypothesis is that both genetic and environmental factors (including chronic alcohol consumption) influence the DNA methylation status of the promoter region of OPRM1, thus changing the expression of MORs or the number of available target sites for occupancy by NTX. Therefore, OPRM1 promoter DNA methylation could influence the efficacy of NTX in AD treatment or moderate the risk of relapse after NTX treatment. In the present study, we investigated the effect of peripheral blood OPRM1 promoter region DNA methylation on AD relapse following NTX treatment in both African American (AA) and European American (EA) subjects with AD. Although blood and brain DNA methylation patterns of OPRM1 may differ because DNA methylation is tissue-specific [32], blood is more easily accessible than brain tissues and thus blood DNA methylation sites may be useful biomarkers of mental disorders, including AD.

**Subjects and methods**

**Study population**

Ninety-three AD subjects (41 AAs and 52 EAs) for the present study were selected from among participants in a study of the effect of opioid receptor gene variants on the outcome of NTX treatment of AD [10]. All subjects were...
male veterans who met criteria for AD on the basis of the Structured Clinical Interview for DSM-IV [33]. They participated in the Veterans Affairs Cooperative Study 425, “Naltrexone in the Treatment of Alcohol Dependence,” a double-blind, placebo-controlled, multicenter NTX treatment trial [34]. To evaluate whether treatment outcome was due to NTX rather than psychological or other factors, we included placebo-treated patients in the study. Table 1 summarizes the demographics and drinking outcomes of the 93 AD subjects included in this pharmacoepigenetic study.

### AD relapse assessment

Outcome variables of NTX or placebo treatment included: (1) number of subjects who relapsed to heavy drinking during the first 13 weeks of treatment [with relapse defined as the first day of heavy drinking (six or more drinks consumed)] [10]; (2) number of days to relapse during the first 13 weeks of treatment; and (3) percent drinking days during the first 13 weeks of treatment.

### OPRM1 promoter region DNA methylation assay

Genomic DNA was extracted from the peripheral blood of the above 93 AD subjects before they initiated NTX or placebo treatment. Genomic DNA (1 μg) was treated with the bisulfite reagent included in the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Two amplicons spanning 734 bp [from 365 bp upstream of the translation start site (TSS) to 369 bp downstream of the TSS] of the OPRM1 promoter region and harboring 44 CpG sites were generated by polymerase chain reactions (PCRs) using two pairs of tagged primers [primers for Amplicon 1: aggaagagagμg] was treated with the bisul

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**Table 1** Subject demographics and alcohol relapse during the first 13 weeks after naltrexone or placebo treatment.

|                         | Africa Americans (n = 41) | European Americans (n = 52) |
|-------------------------|---------------------------|-----------------------------|
|                         | Naltrexone | Placebo | Naltrexone | Placebo |
| Number of subjects      | 28         | 13      | 36         | 16      |
| Age, years (Mean ± S.D.)| 51 ± 9     | 50 ± 11 | 50 ± 10    | 50 ± 11 |
| Naltrexone vs. Placebo  | t = 0.26, df = 1, P = 0.798 | t = 0.07, df = 1, P = 0.943 |
| No. of subjects relapsed| 8 (28.6%)  | 5 (38.5%)| 18 (50.0%) | 8 (50.0%)|
| Naltrexone vs. Placebo  | χ² = 0.40, P = 0.526 | χ² = 0.00, P = 1.000 |
| No. of days to relapse (Mean ± S.D.) | 73 ± 32 | 69 ± 34 | 55 ± 42 | 51 ± 41 |
| Naltrexone vs. Placebo  | t = 0.42, df = 1, P = 0.668 | t = 0.33, df = 1, P = 0.739 |
| Percent drinking days (Mean ± S.D.) | 3.7 ± 5.4% | 11.6 ± 18.5% | 17.1 ± 24.4% | 18.9 ± 30.0% |
| Naltrexone vs. Placebo  | t = −2.10, df = 1, P = 0.051 | t = −0.23, df = 1, P = 0.818 |

Naltrexone: subjects received naltrexone treatment (50 mg/day) for 3–12 months.
Placebo: subjects received matched placebo treatment for 12 months.
−10CpG) are in the 5′ untranslated region (5′ UTR) of OPRM1. 18 CpGs (+12CpG, +23CpG, +27CpG, +53CpG, +84CpG, +126CpG, +135CpG, +140CpG, +145CpG, +150CpG, +159CpG, +182CpG, +186CpG, +206CpG, +215CpG, +237CpG, +243CpG, and +258CpG) are located in the translated region of OPRM1 Exon 1, and four CpGs (+301CpG, +312CpG, +316CpG, and +328CpG) were located in OPRM1 Intron 1 following Exon 1. The methylation calls were performed using the EpiTyper software v1.0 (Sequenom, San Diego, CA), which generated quantitative data (methyl CpG/total CpG) for each CpG site. For CpG sites that were too close to be cleaved apart by RNase A, they were measured as a unit. The methylation levels of four CpGs (CpG +197 or Unit 6, CpG +23 or Unit 17, CpG +237 or Unit 28, and CpG +243 or Unit 29) were not detectable. The remaining 40 CpGs formed 29 CpG units (18 in Amplicon 1 and 11 in Amplicon 2) (Fig. 1).

**Statistical analysis**

Because of the existence of quasi-complete separation [36] in our data set, we used a Bayesian approach to logistic regression to examine the effect of OPRM1 promoter region DNA methylation on patients’ response to NTX or placebo treatment [bayesglm(formula = AD relapse ~ Race + Age + Treatment + Methylation * Treatment, family = binomial(link = "logit")]. Bayesian logistic regression sets an informative prior distribution for the coefficients to be constrained. The analysis was conducted using the R package ‘arm’ version 1.10-1 (https://CRAN.R-project.org/package = arm). The dependent variable was the AD relapse status in the first 13 weeks after at least three months’ of treatment with NTX or placebo. Twenty-nine Bayesian logistic regression models (corresponding to 29 OPRM1 promoter region CpG units) were fitted, each including the same three input variables (race, age, and treatment) and differing by a single input variable (i.e., the methylation level of each of 29 CpG units).

The Random Forest algorithm was used to select a group of input variables as predictors of AD relapse. Because the methylation levels of CpGs in OPRM1 promoter region may be correlated, a regression model assuming that input variables are independent from one another is not suitable for selecting predictors of AD relapse. In the prediction analysis, we used Random Forest, a nonparametric model that does not make any assumptions regarding the structure of the data [37]. The Random Forest analysis has been applied in disease prediction using methylated-CpG sites. For example, Quraishi et al. utilized Random Forest to identify 140 CpG sites that were potentially associated with eczema through the out-of-bag (OOB) error rate calculation [38].

A Random Forest consists of multiple classification trees and each tree is constructed on randomly selected samples that form the training set. The samples that are not in the training set are defined as out-of-bag (OOB) samples. For the same sample, each tree outputs one class and the forest predicts the mode of the class. To compute the OOB error rate [39], the forest first predicts the class of each sample using only the trees that do not contain that sample in the training set to achieve OOB prediction. By comparing OOB prediction with the real class of each sample, the forest outputs the OOB error rate.

When fitting a Random Forest model for a classification task, there are two different measures of variable importance [40]. One is Gini Index—the sum of decreases in node impurities from splitting on the variable, averaged over all trees. Another is Mean Decrease Accuracy—the sum of decreases in prediction accuracy from permuting the variable, averaged over all trees. Variables of low importance can be excluded from a model, making it simpler and faster to fit and predict. Because we were more interested in the accuracy of prediction than the node purity, we used the

![Fig. 1 44 CpG sites in the OPRM1 promoter region (734 bp). Amplicon 1: 27 CpG sites (or 20 CpG units); Amplicon 2: 17 CpG sites (13 CpG units).](image-url)
Bayesian logistic regression analysis results in African Americans (AAs).

| Cpg units | Age | NTX treatment | Cpg methylation | Treatment x Cpg methylation |
|-----------|-----|--------------|-----------------|-----------------------------|
|           | β estimate | P value | β estimate | P value | β estimate | P value | β estimate | P value |
| Unit 1    | −0.05 | 0.256 | −0.40 | 0.606 | −0.75 | 0.384 | 0.55 | 0.644 |
| Unit 2    | −0.05 | 0.231 | −0.47 | 0.550 | 0.01 | 0.991 | 0.00 | 0.999 |
| Unit 3    | −0.05 | 0.232 | −0.35 | 0.654 | −1.76 | 0.170 | 1.27 | 0.382 |
| Unit 4    | −0.06 | 0.197 | −0.48 | 0.535 | −0.17 | 0.881 | −0.59 | 0.649 |
| Unit 5    | −0.07 | 0.131 | −0.75 | 0.364 | 0.83 | 0.429 | −2.67 | 0.171 |
| Unit 6    | −0.05 | 0.232 | −0.48 | 0.529 | 0.04 | 0.973 | −0.04 | 0.975 |
| Unit 7    | −0.06 | 0.217 | −0.95 | 0.296 | 3.02 | 0.080 | −5.52 | 0.034 |
| Unit 8    | −0.05 | 0.218 | −0.43 | 0.578 | −0.91 | 0.448 | 0.75 | 0.596 |
| Unit 9    | −0.06 | 0.218 | −0.47 | 0.545 | 0.70 | 0.404 | 1.62 | 0.194 |
| Unit 10   | −0.05 | 0.232 | −0.51 | 0.504 | −0.17 | 0.803 | 1.25 | 0.255 |
| Unit 11   | −0.05 | 0.278 | −0.61 | 0.428 | 1.02 | 0.371 | −1.30 | 0.320 |
| Unit 12   | −0.05 | 0.232 | −0.46 | 0.550 | −0.35 | 0.798 | 0.34 | 0.852 |
| Unit 13   | −0.05 | 0.232 | −0.35 | 0.654 | −1.76 | 0.170 | 1.27 | 0.383 |
| Unit 14   | −0.06 | 0.194 | −0.34 | 0.665 | 0.54 | 0.381 | 0.25 | 0.847 |
| Unit 15   | −0.05 | 0.253 | −0.51 | 0.504 | 0.01 | 0.996 | 0.52 | 0.647 |
| Unit 16   | −0.05 | 0.277 | −0.61 | 0.427 | 1.03 | 0.367 | −1.30 | 0.319 |
| Unit 17   | −0.06 | 0.196 | −0.45 | 0.566 | −0.92 | 0.274 | 1.53 | 0.186 |
| Unit 18   | −0.05 | 0.231 | −0.61 | 0.451 | −0.19 | 0.883 | −1.69 | 0.352 |
| Unit 19   | −0.06 | 0.219 | −0.30 | 0.706 | −0.71 | 0.571 | −0.43 | 0.736 |
| Unit 20   | −0.05 | 0.198 | −0.64 | 0.424 | −1.38 | 0.200 | −0.30 | 0.817 |
| Unit 21   | −0.05 | 0.238 | −0.37 | 0.634 | −0.53 | 0.666 | −0.11 | 0.927 |
| Unit 22   | −0.05 | 0.225 | −0.42 | 0.584 | −0.15 | 0.859 | −0.31 | 0.780 |
| Unit 23   | −0.06 | 0.172 | −0.57 | 0.489 | 1.23 | 0.160 | −0.33 | 0.768 |
| Unit 24   | −0.06 | 0.167 | −0.40 | 0.614 | 1.02 | 0.239 | −0.18 | 0.868 |
| Unit 25   | −0.05 | 0.265 | −0.57 | 0.469 | 0.32 | 0.815 | −0.69 | 0.644 |
| Unit 26   | −0.06 | 0.211 | −0.52 | 0.503 | −0.10 | 0.923 | 0.54 | 0.603 |
| Unit 27   | −0.05 | 0.225 | −0.42 | 0.584 | −0.15 | 0.859 | −0.31 | 0.780 |
| Unit 28   | −0.06 | 0.177 | −0.40 | 0.622 | 0.97 | 0.212 | −0.59 | 0.582 |
| Unit 29   | −0.07 | 0.142 | −0.41 | 0.610 | 1.37 | 0.494 | 1.26 | 0.632 |

Each row corresponds to a logistic regression model with three variables (age, naltrexone or placebo treatment, and the methylation level of one of the 29 CpG units). The methylation levels of four CpG units (Unit 6 or CpG − 197, Unit 17 or CpG + 23, Unit 28 or CpG + 237, and Unit 29 or CpG243) were not detectable.

Mean Decrease Accuracy to measure the importance of variables to AD relapse. We examined the OOB error rates for Random Forests with a series (1−32) of variables (race, age, treatment, and 29 CpG units ranked by their importance to AD relapse) being used to construct Random Forests. Then we extracted and plotted the prediction error rate of each forest.

Results

AD relapse after NTX or placebo treatment

The relapse information of AD patients during the first 13 weeks after NTX or placebo treatment is summarized in Table 1. Among the 41 AA AD patients, 28 (age ± mean: 51 ± 9 years) received NTX treatment and 13 (age ± mean: 50 ± 9 years) received placebo treatment. Among the 52 EA AD patients, 36 (age ± mean: 50 ± 10 years) received NTX treatment and 16 (age ± mean: 50 ± 11 years) received placebo treatment. The number of patients who relapsed (P > 0.05 by Chi-square tests), the number of days to relapse (P > 0.05 by t-tests), and the percent drinking days (P > 0.05 by t-tests) in the first 13 weeks after the initiation of NTX or placebo treatment did not differ significantly between NTX and placebo treatment groups in either AAs or EAs.

DNA methylation differences between NTX and placebo treatment groups

DNA methylation levels of 29 CpG units (formed by 40 CpG sites) in OPRM1 promoter region were compared between AD patients receiving NTX treatment and those AD patients receiving placebo treatment. As shown in Supplementary Table S1, none of the 29 CpG units had significant differences in their methylation levels between NTX and placebo treatment groups in either AAs or EAs. In other words, these two groups of AD patients had similar
DNA methylation patterns in their OPRM1 promoter regions before receiving NTX or placebo treatment.

**Effect of individual CpG methylation on AD relapse by Bayesian logistic regression analysis**

Methylation of CpG units, NTX treatment, and treatment-by-methylation interactions did not significantly affect the probability of relapse in either AAs or EAs (Tables 2 and 3). However, the effect of age differed between populations, with AAs showing no effect of age, while in EAs, the $P$ values for age were $<0.05$ in all 29 regression models, such that older EA subjects were less likely to relapse ($−0.08 < \beta < −0.07$, $0.017 < P < 0.040$) (Table 3). When both AA and EA subjects were considered, no other variables other than age significantly affected the probability of AD relapse rate (Supplementary Table S2).

**Identification of AD relapse predictors by random forest analysis**

We used the Random Forest algorithm to ascertain the impact of 32 variables (race, age, NTX or placebo treatment, and 29 CpG units; all of the data were presented in Supplementary Table S3) on AD relapse risk. As shown in Fig. 2, age had the highest importance to AD relapse. This finding was consistent with the output of the Bayesian logistic regression model, which showed that age was the only variable that significantly influenced the risk of relapse. In addition, we used the Random Forest algorithm to select a group of variables from the above 32 variables that could predict relapse with the highest accuracy (or the lowest error rate). As indicated in Fig. 3, the lowest prediction error rate (34%) [or the highest prediction rate (66%)] was achieved when the top five most important variables (age and methylation levels at Unit 3, Unit 8, Unit 14, and Unit 15) were used to fit the Random Forest model as predictors.

**Discussion**

Although NTX is FDA-approved for treating AD, the responsiveness of AD patients to NTX treatment is highly variable. Pharmacogenetic studies have investigated whether the heterogeneity in NTX’s treatment effects is due to variation in opioid receptor genes, but the findings have been inconsistent. The present study tested the hypothesis that DNA methylation patterns in the OPRM1 promoter region moderated AD patients’ response to NTX treatment. Our data did not reveal a significant effect of methylation of individual CpG units in the OPRM1 promoter region on relapse to heavy drinking after NTX or placebo treatment, although age and a group of CpG units may influence AD relapse in an integrative manner.

There are several possible explanations for the negative findings. First, our sample size was small. It provided limited statistical power to identify CpGs with a moderate effect on AD relapse. Second, cell heterogeneity from blood samples may bias the results. DNA methylation variation resulting from different proportions of blood cell types may mask the response difference among AD patients receiving NTX or placebo treatment. Recent studies have shown that peripheral blood DNA methylome profiles (or methylation levels of a set of CpGs in the genome) could be used as biomarkers to infer the proportions of different types of blood cells, including CD8+ and CD4+ T-lymphocytes, natural killer cells, B cells, monocytes, and granulocytes [41, 42]. In future studies, when DNA methylome data are available for subjects included in the present study, we will be able to control for the potential cofounding effects of blood cell types by taking estimated cellular proportions into consideration. Third, although DNA methylation changes in blood can serve as useful biomarkers for health or disease status, DNA methylation patterns of genes in peripheral blood may be distinct from those in the brain. The µ-opioid receptor (coded by OPRM1) is primarily expressed in the brain, where it mediates the rewarding effects of opioids and other drugs of abuse by modulating the dopamine system [43]. However, OPRM1 is also expressed in white blood cells, and peripheral blood OPRM1 DNA methylation levels are potential biomarkers of AD severity and treatment outcome. In addition, DNA methylation in the OPRM1 promoter region may not have a major regulatory effect on MOR expression in the brain, as demonstrated in the study by Knothe et al. [22]. Thus, there may not be a measurable effect of OPRM1 DNA methylation status on NTX treatment outcome.

It is intriguing that age had a significant effect on AD relapse in EAs, among whom older subjects had a lower relapse rate. Previous studies also demonstrated that older age was associated with better outcomes for alcohol and other drug addiction treatment (using nonpharmacological therapies, such as supportive group therapy, education, relapse prevention, and family-oriented therapy) [44]. Accumulating evidence suggests that DNA methylation changes are highly correlated with chronological age in human brains [45]. Our finding presumably reflects the effects of aging on the epigenetic status of rewarding or addiction-related genes, making older subjects more responsive to pharmacological treatment. In addition, the interaction of age and genetic variants (e.g., OPRM1 SNP A118G) could influence the NTX treatment response. Thus, we genotyped A118G in EA AD patients. It showed that a greater proportion (25.0%) of older EAs (above the mean
Table 3 Bayesian logistic regression analysis results in European Americans (EAs).

| Age          | NTX treatment | CpG methylation | Treatment x CpG Methylation |
|--------------|---------------|-----------------|----------------------------|
|              | β estimate    | P value         | β estimate                 | P value         | β estimate                 | P value         |
| Unit 1       | -0.07         | 0.025           | 0.12                       | 0.858           | 0.45                       | 0.580           | -0.66  | 0.544 |
| Unit 2       | -0.07         | 0.031           | 0.03                       | 0.957           | 0.82                       | 0.369           | -0.48  | 0.633 |
| Unit 3       | -0.07         | 0.026           | 0.07                       | 0.917           | -0.01                      | 0.989           | 0.02   | 0.977 |
| Unit 4       | -0.07         | 0.027           | 0.07                       | 0.911           | -0.27                      | 0.795           | 0.51   | 0.631 |
| Unit 5       | -0.07         | 0.026           | 0.11                       | 0.870           | 0.38                       | 0.618           | -0.45  | 0.603 |
| Unit 6       | -0.07         | 0.027           | 0.13                       | 0.847           | -0.46                      | 0.553           | 0.56   | 0.537 |
| Unit 7       | -0.07         | 0.026           | 0.02                       | 0.974           | 0.13                       | 0.867           | 0.31   | 0.721 |
| Unit 8       | -0.07         | 0.034           | 0.05                       | 0.942           | 0.54                       | 0.435           | -1.54  | 0.151 |
| Unit 9       | -0.08         | 0.018           | 0.21                       | 0.748           | 1.79                       | 0.091           | -1.49  | 0.195 |
| Unit 10      | -0.07         | 0.025           | 0.13                       | 0.845           | 0.30                       | 0.687           | 0.02   | 0.988 |
| Unit 11      | -0.07         | 0.025           | 0.07                       | 0.919           | -0.12                      | 0.887           | 0.27   | 0.778 |
| Unit 12      | -0.08         | 0.017           | 0.08                       | 0.896           | -1.12                      | 0.216           | 1.19   | 0.218 |
| Unit 13      | -0.07         | 0.026           | 0.07                       | 0.917           | -0.01                      | 0.989           | 0.03   | 0.976 |
| Unit 14      | -0.07         | 0.032           | -0.01                      | 0.989           | -1.56                      | 0.176           | 1.26   | 0.326 |
| Unit 15      | -0.07         | 0.029           | 0.16                       | 0.812           | -0.76                      | 0.433           | 0.58   | 0.574 |
| Unit 16      | -0.07         | 0.026           | 0.07                       | 0.919           | -0.12                      | 0.889           | 0.27   | 0.781 |
| Unit 17      | -0.07         | 0.026           | 0.13                       | 0.848           | 0.19                       | 0.842           | -1.33  | 0.228 |
| Unit 18      | -0.07         | 0.035           | 0.01                       | 0.984           | -0.35                      | 0.747           | 0.29   | 0.820 |
| Unit 19      | -0.07         | 0.037           | 0.06                       | 0.929           | 0.00                       | 0.998           | -0.11  | 0.925 |
| Unit 20      | -0.07         | 0.027           | 0.02                       | 0.977           | 0.15                       | 0.844           | 0.39   | 0.684 |
| Unit 21      | -0.08         | 0.023           | 0.07                       | 0.920           | -0.05                      | 0.960           | 0.60   | 0.579 |
| Unit 22      | -0.08         | 0.023           | 0.27                       | 0.688           | -0.75                      | 0.498           | 1.49   | 0.217 |
| Unit 23      | -0.07         | 0.033           | 0.06                       | 0.931           | 1.19                       | 0.252           | -0.50  | 0.664 |
| Unit 24      | -0.07         | 0.026           | 0.08                       | 0.901           | -0.13                      | 0.889           | 0.10   | 0.926 |
| Unit 25      | -0.07         | 0.024           | 0.09                       | 0.888           | 0.29                       | 0.689           | -0.04  | 0.964 |
| Unit 26      | -0.07         | 0.028           | 0.11                       | 0.871           | -0.76                      | 0.481           | 1.08   | 0.349 |
| Unit 27      | -0.08         | 0.023           | 0.27                       | 0.689           | -0.75                      | 0.499           | 1.48   | 0.217 |
| Unit 28      | -0.07         | 0.040           | 0.24                       | 0.720           | -1.31                      | 0.241           | 1.64   | 0.199 |
| Unit 29      | -0.08         | 0.022           | 0.03                       | 0.969           | -0.10                      | 0.904           | 0.53   | 0.535 |

Each row corresponds to a logistic regression model with three variables (age, naltrexone or placebo treatment, and the methylation level of one of the 29 CpG units). The methylation levels of four CpG units (Unit 6 or CpG 237, Unit 17 or CpG + 23, Unit 28 or CpG + 237, and Unit 29 or CpG243) were not detectable.

Age of 50 years) had genotype A/G compared with younger EAs (less than 50 years old) (8.3%). However, further studies are warranted to validate the finding and explore the mechanism by which the effects of DNA methylation or genetic variation occur.

A major limitation of the present study is the lack of availability of blood for DNA extraction obtained after treatment. This would have allowed us to test another hypothesis, i.e., that NTX treatment alters the DNA methylation patterns of reward or addiction-related genes such as OPRM1, leading to altered expression of these genes and a higher level of responsiveness (or no relapse within 13 weeks) to NTX treatment. As shown in Table 1, some AD subjects did not relapse during the 13-week treatment period. It is thus of interest to understand why these AD subjects were more responsive to NTX treatment than others. It is unknown whether NTX or placebo treatment alters epigenetic status of genes (including OPRM1), resulting in a better outcome (or no AD relapse) after treatment. Follow-up epigenome-wide associated studies (EWAS) can determine whether DNA methylation patterns are changed in certain genes after NTX or placebo treatment. In addition, it is unknown whether brain tissue OPRM1 promoter DNA methylation exerts a significant effect on AD relapse, but brain tissue sample is not accessible.

Efforts to improve the efficacy of pharmacotherapy for AD through the use of personalized approaches can help in avoiding the exposure to medications of patients who are unlikely to respond to them. One approach for realizing a precision approach to treating AD involves pharmacogenetic studies that can be used to select a subgroup of patients who are most likely to benefit from the treatment. Another approach involves pharmacopregeenetic studies, in which patients' epigenetic status (e.g., DNA methylation levels) is used to match them with specific pharmacotherapies that optimize the response to treatment. Despite the lack of a significant finding of an epigenetic moderator
of NTX response, the present study provides a useful initial effort and a model for subsequent research using this approach. In our future pharmacogenetic studies, we may consider subgrouping patients based on methylation levels (high vs. low) of specific promoter CpG sites (particularly those CpGs showing differential methylation in patients and located in transcription factor binding sites) before treatment, and then we analyze whether treatment outcome is different between these two subgroups of patients. In this way, we can directly examine the impact of OPRM1 promoter CpG methylation on treatment outcome.

Acknowledgements We thank all participants in this study. We are grateful to Dr Joel Gelernter and his colleagues at Yale University School of Medicine for providing DNA samples and clinical data for the conduct of this study.

Compliance with ethical standards

Conflict of interest This work was supported by grants (R21AA023068 and R01AA025080) from the National Institute on Alcohol Abuse and Alcoholism. HRK is a member of the American Society of Clinical Psychopharmacology’s Alcohol Clinical Trials Initiative (ACTIVE), which during the past three years was supported by AbbVie, Allergen, Amygdala Neurosciences, Arbor Pharmaceuticals, Ethypharm, Indivior, Lilly, Lundbeck, Otsuka, and Pfizer. HRK is named as an inventor on PCT patent application #15/878,640 entitled: “Genotype-guided dosing of opioid agonists,” filed January 24, 2018. All other authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of this paper.

Ethical approval Ethics statements: The study was exempted from a specific ethical approval by Boston University School of Medicine in accordance with local/national guidelines. This study only involved DNA methylation data analysis, and the de-identified DNA samples were from our collaborator Dr Joel Gelernter at Yale University School of Medicine. Patient samples and demographic information were collected as part of previous studies (Krystal et al. [34]; Gelernter et al. [10]).

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Fig. 2 Importance of variables to relapse estimated by the Random Forest algorithm. The plot shows 32 variables (age, race, naltrexone treatment, and 29 CpG units) on the Y-axis, and their importance to relapse to heavy drinking on the X-axis. The variables are ordered top-to-bottom as most- to least-important to relapse to heavy drinking.

Fig. 3 Out-of-bag (OOB) error rates versus numbers of most important variables to relapse included in the Random Forest prediction model. The plot shows cumulative OOB error rates (on the Y-axis) as a function of numbers (on the X-axis) of most important variables (age, race, treatment, and 29 CpG units; ranked from most- to least-important to relapse to heavy drinking) included in the Random Forest prediction model.
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