



## Short communication

DNA methylation at *DLGAP2* and risk for relapse in alcohol dependence during acamprosate treatment

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## ABSTRACT

**Background:** Alcohol use disorders are prevalent mental disorders with significant health implications. Epigenetic alterations may play a role in their pathogenesis, as DNA methylation at several genes has been associated with these disorders. We have previously shown that methylation in the *DLGAP2* gene, coding for a synaptic density protein, is associated with alcohol dependence. In this study, we aimed to examine the association between *DLGAP2* methylation and treatment response among patients undergoing acamprosate treatment.

**Methods:** 102 patients under acamprosate treatment were included. DNA methylation analysis at *DLGAP2* was performed by bisulfite pyrosequencing at the start and after 3-month treatment. Treatment outcomes were having a relapse during the treatment and severity of craving at the end of three months. Cox proportional hazard and linear regression models were performed.

**Results:** Patients whose methylation levels were decreased during the treatment showed an increased risk for relapse within three months in comparison to the ones without methylation change (hazard ratio [HR]=2.44; 95% confidence interval [CI]=1.04, 5.73; p=0.04). For the same group, a positive association for the severity of craving was observed, yet statistical significance was not reached ( $\beta$ =2.97; 95% CI=-0.41, 6.34; p=0.08).

**Conclusion:** We demonstrate that patients whose *DLGAP2* methylation levels decrease during acamprosate treatment are more likely to relapse compared to the ones without changes. This is in line with our previous findings showing that *DLGAP2* methylation is lower in alcohol dependent subjects compared to controls, and might suggest a role for changes in *DLGAP2* methylation in treatment response.

## 1. Introduction

Alcohol use disorders are one of the most common mental disorders and associated with a high disease burden, including disability, medical comorbidities, and increased mortality (Carvalho et al., 2019). A significant proportion of the patients with alcohol use disorders do not receive any kind of therapeutic interventions (Carvalho et al., 2019),

and identifying patients who would benefit from treatment options available is challenging due to the lack of biomarkers. Furthermore, our knowledge regarding the biological underpinnings of alcohol use disorders as well as mechanisms of medical treatments in use is rather limited despite the high prevalence and devastating consequences of these disorders.

Both genetic and environmental factors contribute to the

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development of alcohol use disorders (Goldman et al., 2005). Epigenetics has been of particular interest to better understand the underlying pathogenesis. This is due to the fact that epigenetic modifications, including DNA methylation, regulate gene expression and are determined by environmental stimuli as well as by the genome. DNA methylation alterations in various genes have been associated with alcohol use disorders as well as respective clinical parameters (Hillmacher et al., 2019; Witt et al., 2020; Longley et al., 2021; Dragic et al., 2022; Zhao et al., 2022). We previously showed that DNA methylation at the *DLGAP2* gene, which encodes a postsynaptic density protein (Takeuchi et al., 1997), was associated with alcohol dependence, and that the association was influenced by genotype (Meng et al., 2021). In the same study, lower methylation at *DLGAP2* was also associated with an increased frequency of drunkenness in adolescents without alcohol dependence. Considering that genetic variants of *DLGAP2* have been shown to play roles in other psychiatric disorders, including schizophrenia, autism spectrum disorder, and dementia (Rasmussen et al., 2017; Ouellette et al., 2020), it might be an important target to better understand alcohol use disorders. Yet, the role of *DLGAP2* in these disorders remains largely unknown.

In this study, we aimed to assess the associations between the DNA methylation status at the *DLGAP2* gene and treatment response to one of the first-line treatment options, acamprosate, in patients with alcohol dependence. Based on our previous results, we hypothesized that there might be an inverse association between *DLGAP2* methylation levels and relapse and craving for alcohol use after three months of acamprosate treatment.

## 2. Methods

### 2.1. Study participants and design

This study was utilizing DNA samples and clinical information from a subset of subjects enrolled in a prospective study conducted among alcohol-dependent subjects treated with acamprosate in community-based programs (Karpyak et al., 2014). All participants were interviewed with PRISM by trained and certified interviewers, and they met Diagnostic and Statistical Manual for Mental Disorders, 4th edition (DSM-IV) criteria for alcohol dependence (Hasin et al., 1996). Exclusion criteria were: not being able to provide informed consent or speak English, having unstable physical (e.g. renal or hepatic impairment) or psychiatric (e.g. psychotic disorder or active suicidal ideation) conditions, pregnancy or breastfeeding, planning pregnancy within the follow-up period, using disulfiram, and contraindication for acamprosate treatment (e.g. previous allergic reaction). The current study consists of 102 participants with complete data on DNA methylation, treatment outcomes after 3-month follow-up, and covariates.

All participants provided informed consent, and the study was approved by the Institutional Review Boards of the Mayo Clinic Rochester and Mayo Clinic Health System.

### 2.2. Genotyping and DNA methylation analysis

#### 2.2.1. SNP genotypes were coded using an additive minor allele dosage model

DNA methylation analysis of the CpG site cg05041795 (chr8:1365749–1365749, hg19) within DMR-*DLGAP2* was performed by bisulfite pyrosequencing. Shortly, DNA was bisulfite converted using the EZ-96 DNA Methylation-Gold MagPrep from Zymo Research (Irvine, California, USA) according to the manufacturer's instructions. The target region was amplified using the PyroMark PCR Kit (Qiagen), forward primer (TGTGAAGGTTAATGTGGTAATAGTT, Integrated DNA Technologies [IDT]), biotinylated reverse primer (AAAATCCTCTAAACCTCTATTATC, Biomers GmbH), and approximately 10 ng of converted DNA, following the manufacturer's instructions. Pyrosequencing was performed on a PyroMark Q24 with 0.3  $\mu$ M sequencing

primer (GGAGGAGAGGAGAAAAT, IDT) according to the manufacturer's protocol. Percentage methylation was calculated using the PyroMark Q24 2.0.8 software (Qiagen). Linearity of the assay was tested using CpGenome™ Human Methylated DNA Standard Set and CpGenome™ Human Non-Methylated DNA Standard Set (Sigma - Merck).

### 2.3. Treatment outcomes

Relapse was defined as any amount of alcohol consumption during the 3-month treatment period. No relapse vs. relapse was determined by self-reports, and blood gamma-glutamyl transpeptidase (GGT) measurements were used to assess the accuracy of self-reports.

To assess craving due to alcohol dependence, the Penn Alcohol Craving Scale (PACS) was used at the beginning of the follow-up and after three months (Flannery et al., 1999). PACS is a self-report questionnaire consisting of 5 items with a total score range from 0 to 30; higher scores indicate more craving.

### 2.4. Covariates

Age, sex, research site, smoking status, and comorbid depression were included as covariates. Research sites where participants were recruited were categorized in five groups. Smoking status was defined with three categories which are never, former and current smokers. Comorbid depression was assessed with a semi-structured interview, PRISM.

### 2.5. Statistical analysis

The relation between genotype and DNA methylation levels was assessed using linear regression analysis. Each SNP variable included three categories as major homozygote, heterozygote, and minor homozygote alleles.

To investigate the association between DNA methylation at *DLGAP2* and relapse after 3-month acamprosate treatment, Cox proportional hazard model was employed. Crude models with no adjustment and fully adjusted models with adjustment for age, sex, research site, smoking status, and comorbid depression were built. The association between *DLGAP2* methylation and craving after 3-month acamprosate treatment was examined using linear regression analysis. The crude models were only adjusted for baseline PACS score, while the adjusted models were built with the abovementioned covariates. In these analyses, DNA methylation was modelled as either continuous or categorical variables. Continuous DNA methylation variables were *baseline* methylation level, methylation level *after 3-month treatment* and *delta* methylation computed by subtracting baseline level from 3-month level. By using the coefficient of variation of methylation change, delta methylation was also transformed into a categorical variable, defined as *decreased*, *increased* or *no change* in methylation after 3-month treatment compared to baseline methylation. To assess the stability of the results, the analyses concerning DNA methylation status and treatment outcomes were bootstrapped by constructing 10,000 of resamples with replacement.

All analyses were performed using R version 4.2.1.

## 3. Results

Characteristics of the study population are presented in Table 1. The mean age was 44.1 years (standard deviation [SD]= 12.5), and 65 participants (63.7%) were men. Majority of the participants were white (97.1%) and non-Hispanic or Latino (93.1%). Out of 102, 33 (32.4%) participants had relapses within three months. The median PACS score was 13 (interquartile range [IQR]= 12.7) at baseline and 5 (IQR= 7) during 3-month follow-up assessment. The number of non-relapsed and relapsed participants in each group and baseline and 3-month methylation levels for those are depicted in Supplementary Figure S1.

**Table 1**  
Descriptive characteristics of the study participants (N=102).

	Mean ± SD N, (%) Median [IQR]
Age, years	44.1 ± 12.5
Men	65 (63.7)
White	99 (97.1)
Not Hispanic or Latino	95 (93.1)
Research site	
Albert	23 (22.5)
Austin	10 (9.8)
FSH	18 (17.7)
IAP/OAs	31 (30.4)
Others	20 (19.6)
Smoking	
Never smoked	21 (20.6)
Former smoker	12 (11.8)
Current smoker	69 (67.6)
Depressed	22 (21.6)
Methylation	
Baseline	91.2 [5.7]
3-month	91.7 [7.5]
Methylation change groups	
No methylation change	47 (46.1)
Decreased methylation	27 (26.5)
Increased methylation	28 (27.4)
PACS score (N=90)	
Baseline	13 [12.7]
3-month	5 [7]
Relapsed	33 (32.4)
Days until the first drinking	90 [28.7]
Days until the heavily first drinking	90 [19.5]

In our previous study (Meng et al., 2021), DNA methylation at a region upstream of the *DLGAP2* gene (*DLGAP2*-DMR) was shown to be dependent on genotype. In a first step, we investigated the associations between these SNPs and DNA methylation levels using linear regression analysis in our cohort. The previous findings were confirmed for 22 out of the 30 SNPs where genotype was significantly related to levels of baseline methylation (Supplementary Table S1).

We then addressed whether changes in *DLGAP2* methylation during acamprosate treatment are associated with treatment outcomes. Our analyses from fully adjusted models showed that participants with decreased DNA methylation at *DLGAP2* under acamprosate treatment were more likely to have relapse during 3-month treatment in comparison to participants with no DNA methylation change (Hazard ratio [HR]= 2.44; 95% confidence interval [CI]= 1.04, 5.73; p= 0.04; Table 2). When using the continuous values for methylation, there was no significant association of baseline, 3-month or delta methylation levels with the likelihood of relapse. Similarly, in the analyses with PACS score as outcome, we did not observe any significant association between baseline, 3-month or delta methylation levels. However, the risk

**Table 2**  
Associations between *DLGAP2* methylation and acamprosate treatment outcomes.

	Relapse within three months (N=102)				PACS score after three months (N=90)			
	Model-1		Model-2		Model-1		Model-2	
	HR (95% CI)	p value	HR (95% CI)	p value	beta (95% CI)	p value	beta (95% CI)	p value
Baseline methylation	1.05 (0.97, 1.13)	0.22	1.03 (0.96, 1.12)	0.40	-0.09 (-0.34, 0.16)	0.48	-0.11 (-0.37, 0.16)	0.43
3-month methylation	1.02 (0.95, 1.09)	0.66	1.01 (0.94, 1.08)	0.78	-0.15 (-0.39, 0.09)	0.22	-0.17 (-0.43, 0.08)	0.18
Delta methylation	0.93 (0.84, 1.03)	0.17	0.96 (0.87, 1.06)	0.44	-0.19 (-0.61, 0.23)	0.37	-0.22 (-0.67, 0.23)	0.33
Delta methylation groups								
Group 1 <sup>a</sup>	<b>2.87 (1.30, 6.33)</b>	<b>0.009</b>	<b>2.44 (1.04, 5.73)</b>	<b>0.04</b>	2.61 (-0.52, 5.74)	0.10	2.97 (-0.41, 6.34)	0.08
Group 2 <sup>b</sup>	1.24 (0.50, 3.09)	0.64	1.01 (0.39, 2.63)	0.98	0.16 (-2.79, 3.11)	0.91	0.25 (-3.01, 3.51)	0.88

PACS, the Penn Alcohol Craving Scale; HR, hazard ratio; CI, confidence interval. Model-1 is unadjusted for relapse and only adjusted for baseline PACS score for craving. Model-2 is additionally adjusted for age, gender, research site, smoking, depression for both outcomes.  
<sup>a</sup>Group 1 consists of participants with decreased methylation after three months.  
<sup>b</sup>Group 2 consists of participants with increased methylation after three months.

for higher PACS scores in the group of individuals with decreased *DLGAP2*-DMR methylation compared to no methylation change during acamprosate treatment was close to the statistical significance level (β= 2.97; 95% CI= -0.41, 6.34; p= 0.08).

The bootstrapped results showed similar patterns although statistical significance was not reached (Supplementary Tables S2). Still, HR>1 for relapse was observed in 96.4% of resamples in the adjusted models.

#### 4. Discussion

In this study, we examined associations between DNA methylation at the *DLGAP2* gene and relapse and severity of craving after 3-month treatment of acamprosate among patients diagnosed with alcohol dependence. Patients whose methylation levels decreased over the course of treatment showed an increased likelihood of having relapse within three months in comparison to the ones with no methylation changes. Our results for the severity of craving also suggested worse treatment outcomes for the ones with decreased methylation levels, even though those results did not reach the significance level. We also confirmed the previous findings that genotype is related to methylation status of the *DLGAP2* gene.

Our previous findings showed that post-mortem brain samples of patients with alcohol dependence display decreased *DLGAP2* methylation levels compared to healthy controls (Meng et al., 2021). Furthermore, decreased methylation in blood was associated with increased frequency of drunkenness in adolescents of the IMAGEN study without alcohol dependence. Additionally, in cells, *DLGAP2* methylation was inversely correlated with expression of the gene, and *Dlgap2*-deficient mice consumed less alcohol in comparison to wild-type controls in the “two bottle preference” and “drinking in the dark” experiments (Meng et al., 2021). Similar behaviors were shown for *Dlg4* deficient mice (Camp et al., 2011). *DLG4* encodes the postsynaptic density protein 95 (PDS-95), which is an interaction partner of *DLGAP2* protein (Takeuchi et al., 1997). Through intermediary proteins, *DLGAP2* interacts with N-methyl-D-aspartate (NMDA) receptors whose role in alcohol consumption and dependence is well-established (Mira et al., 2019). Therefore, alterations in *DLGAP2* levels might lead to disruptions in NMDA receptor-mediated synaptic transmission. Thus, our findings suggest a functional role for *DLGAP2* in alcohol dependence, similar to its involvement in other psychiatric and neurological disorders (Rasmussen et al., 2017; Ouellette et al., 2020). Yet, in the current study, we did not observe any significant association between baseline or follow-up methylation levels of *DLGAP2* with craving or treatment outcomes. This could be due to the different study designs or relatively small sample size in this current study. However, the finding that decreased methylation under acamprosate treatment is associated with worse clinical outcome is in line with the previous findings, although we cannot exclude a potential effect of the acamprosate treatment itself.

Further mechanistic studies are required to delineate *DLGAP2*'s functional role in alcohol consumption and dependence as well as its potential interrelationships with acamprosate.

The present study has some limitations. We did not investigate DNA methylation changes on brain samples where *DLGAP2* is expressed and active, instead, blood samples were analyzed. However, a high correlation between the methylation levels of *DLGAP2* in the brain and blood was established previously (Meng et al., 2021). Additionally, we cannot exclude a potential impact of cell composition on our findings. Yet, results from a small subset of the study participants indicate that only monocyte count differs between relapsed and non-relapsed participants and that methylation at the *DLGAP2* gene is not correlated to monocyte counts. Our sample size is also relatively small which might have resulted in underestimation of potential associations. On the other hand, confirming our previous results on the relationship between DNA methylation of *DLGAP2* and alcohol dependence in another cohort stands as a prominent strength.

In conclusion, we show that patients whose methylation levels at *DLGAP2* decrease under acamprosate treatment are more likely to have relapse compared to the ones without changes. Our current findings strengthen previous results suggesting a role for *DLGAP2* in alcohol dependence and links those to effects of acamprosate treatment. Further pre- and clinical investigations are required to better understand the role of *DLGAP2* in alcohol dependence and treatment of the disorder.

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#### CRediT authorship contribution statement

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#### Declaration of Competing Interest

None.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at [doi:10.1016/j.drugalcdep.2024.111116](https://doi.org/10.1016/j.drugalcdep.2024.111116).

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