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TOO MUCH ALCOHOL, DIFFERENT GENE SURFACE?

Alcohol abuse could be associated with monoamine oxidase A gene methylation

Research project

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Running head: *MAOA* methylation and alcohol abuse

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Too much alcohol, different gene surface? Alcohol abuse could be associated with monoamine oxidase A gene methylation

Monoamine oxidase A (MAO-A) is an enzyme that metabolizes neurotransmitters involved in alcohol dependency. To investigate if MAO-A is associated with alcohol abuse, I compared *MAOA* gene methylation in males with or without alcohol use disorders (N = 24 and N = 55, respectively). Methylation of *MAOA* gene in white blood cells was used as an index of MAO-A activity in the brain. Average methylation was higher in males with alcohol use disorders (AUD), but only in the nonsmokers' subsample (Wilcoxon p = 0.01, proportion of better responses = 0.86). Methylation at gene site CpG10 was also higher in nonsmokers with AUD (p = 0.05, proportion = 0.80). Interaction between smoking and methylation is a novel finding. Because the sample of nonsmokers is small (N = 5 + 19), these results should be considered only as initial.

Keywords: monoamine oxidase A, DNA methylation, alcohol use disorder

Liigne alkohol – muutused geeni pealispinnas? Alkoholi väärkasutamine võib olla seotud monoamiinoksüdaas A geeni metüleeritusega

Monoamiinoksüdaas A (MAO-A) on ensüüm, mis lagundab alkoholsõltuvusega seotud virgatsaineid. Uurimaks, kas MAO-A on seotud alkoholi väärkasutamisega, võrdlesin *MAOA* geeni metüleeritust alkoholikasutushäirega meestel (N = 24) ja kontrollgrupil (N = 55). *MAOA* geeni metüleeritus valgetes vererakkudes näitab kaudselt MAO-A aktiivsust ajus. Alkoholikasutushäirega meestel oli keskmine metüleeritus kõrgem, kuid ainult siis, kui vaadata eraldi mittesuitsetavaid inimesi (Wilcoxoni p = 0.01, paremate tulemuste osakaal = 0.86). Mittesuitsetajatest alkoholikasutushäirega meestel oli metüleeritus kõrgem ka geenipiirkonnas CpG10 (p = 0.05, osakaal = 0.80). Suitsetamise ja metüleerituse interaktsioon on uudne leid. Kuna mittesuitsetajate valim on väike (N = 5 + 19), peaks neid tulemusi pidama ainult esialgseteks.

Märksõnad: monoamiinoksüdaas A, DNA metülatsioon, alkoholikasutushäire

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1. Introduction

1.1. Background information

Monoamine oxidase (MAO) is an enzyme that catalyzes the breakdown of important neurotransmitters, such as serotonin, epinephrine, norepinephrine and dopamine. MAO exists in two forms – A and B. Form A (MAO-A) is mainly located in catecholaminergic neurons, form B (MAOA-B) is mainly synthesized in serotonergic and histaminergic neurons and astrocytes (as reviewed by Bortolato, Chen & Shih, 2008).

Studies have shown that both dopamine and norepinephrine play an important role in alcohol dependency (Morikawa & Morrisett, 2010; Weinshenker & Schroeder, 2007). Because dopamine and norepinephrine are both related to alcohol dependency and because MAO-A plays a role in their metabolism, we could assume that the activity of MAO-A in the brain is also related to alcohol dependency.

One factor that could affect MAO-A brain activity is the methylation of *MAOA* gene[1]. In a recent study, Shumay, Logan, Volkow and Fowler (2012) found that average methylation of the promoter region of *MAOA* gene in white blood cells is correlated to the activity of MAO-A inside the brain ($r = –0.61$, $p < 0.001$).

Methylation is the addition of methyl groups to the base pairs of DNA, mainly to cytosine in multicellular organisms (Klose & Bird, 2006, p. 89, 91). Methyl group itself consists of one carbon atom and three hydrogens. Methylation could be one of the mechanisms for regulating gene activity, because it is related to gene silencing (Brenet et al, 2011, p. 5–6).

Considering the information above, we can hypothesize that the methylation state of *MAOA* gene in white blood cells is related to alcohol dependency. In a recent study, this relationship was found in women (Philibert, Gunter, Beach, Brody, & Madan, 2008). Philibert and colleagues did not report if methylation was higher or lower. However, Zhang et al. (2013) found in a genome wide study that in people with alcohol dependency, methylation was lower in most gene regions which were differently methylated.

Therefore, I am starting from the idea that methylation is lower in people who use alcohol harmfully.

[1] In this paper, the symbol "MAO-A" refers to monoamine oxidase A enzyme and the symbol "*MAOA*" refers to monoamine oxidase A gene. This is a guideline of the HUGO Gene Nomenclature Committee (Wain et al., 2002).

1.2. Research design and hypotheses

In this study, I am testing the hypothesis that methylation is lower in people who abuse alcohol. For that purpose I am comparing two groups of males – men with or without alcohol use disorders (AUD).

As I am comparing two groups, it is important that the groups would be similar in terms of other variables that can also affect methylation. These variables are gender, age, *MAOA* genotype and smoking (see 2.2). In this data, smoking frequency is not similar in groups. I will therefore use statistical methods to control for smoking. Because of that, it is convenient to structure my analysis around two questions:

1. Is methylation lower in the AUD group?
2. Is methylation lower in the AUD group, when smoking is controlled for?

In addition, it would be interesting to know if there has been more stress in the AUD group. This is important, because methylation could be related to stress. For example, Domschke et al. (2012, p. 1223) found that the occurrence of negative life events in previous 6 or 12 months was associated with decreased methylation at two sites of *MAOA* gene and suggested that methylation could mediate the influence of stressful events on mental disorders. Therefore, if the two groups differ both in methylation and stress, it is *possible* that methylation mediates the relationship between stress and alcohol abuse. To explore this possibility, I will add a third research question:

3. Has there been more stress in the AUD group?

However, the question about stress is not the main focus. Considering the data used for this study, it is not possible to clearly show whether mediation exists or not.[2] Nevertheless, adding this question is still of interest, because it can show whether a mediating relationship is possible.

To explore these three questions, I am analyzing data that has already been collected by the research team of Jaanus Harro in cooperation with the National Institute for Health Development in Tallinn. The details of this data analysis are discussed in section 2 and results are given in section 3.

[2] I considered Kenny's (2014) suggestions for showing mediation and came to the conclusion that there are three reasons why mediation cannot be clearly shown in this study. Firstly, at the moment we do not have longitudinal data for methylation. Only if methylation precedes alcohol abuse in time, can it be a mediator. Secondly, another important property of mediation cannot be shown: when methylation is controlled for, the effect of stress on alcohol abuse should be weaker or nonexistent. This property cannot be shown, because the regression model should have three predictor variables (stress, methylation and smoking), but sample size is too low for having three predictors (n = 24 in the smaller group). Thirdly, because I have a case-control sample for alcohol abuse, I do not have a random sample of people with respect to stress. Random sample for stress is necessary to show the effect of stress on methylation, and for being able to compare the regression equations used for establishing mediation.

1.3. Importance of the study

First, if people with alcohol use disorders differ in methylation, methylation could become a diagnostic marker (as suggested by Zhang et al, 2013, p. 393).

Second, this study could provide additional knowledge about the role of MAO-A in alcohol abuse in humans. I am aware of only one study that has been done on the role of *MAOA* methylation in alcohol dependency (Philibert et al, 2008). There also seems to be a lack of studies looking at the general role of methylation in alcohol dependency – Zhang et al. (2013, p. 393) list 5 studies in the introduction of their own report.

Third, this study could hint at the interplay of gene activity (methylation) and environment (stress). To my knowledge, there are no studies that have looked at both *MAOA* methylation and the number of stressful events. However, this study is not fully suited for showing a relationship between stress and methylation.

2. Method

2.1. Initial data collection and later data selection

Sample. Participants were taken from the Estonian sample of European Youth Heart Study (EYHS), which was later developed into the Estonian Children Personality Behaviour and Health Study (ECPBHS). This study uses the older cohort of EYHS. 37 males who had abused alcohol in their life and 82 males who had not abused alcohol were selected for analysis. People were not recruited separately for this study – data comes from their blood samples and other measures recorded in the database of ECPBHS.

Gender. Only male participants were chosen in order to reduce the complexity of analysis (see 2.2. below).

Measurement waves. In the ECPBHS study, participants were measured three times – in 1998, 2001 and 2008, when they were approximately 15, 18 and 25 years old. Among other measures, their blood samples were obtained. For the purposes of this study, *MAOA* gene methylation was measured from blood samples collected in 1998, 2001 and 2008. Blood samples were put into four plates; two plates were analyzed at one time and two plates were analyzed at a later time.

Choice of data for this study. The quality of methylation data was found to be unclear, because blood samples that were analyzed at a later time gave significantly lower results. This suggested that analysis conditions may not have been equal. Considering this, as well as considering size limits of this paper, I report results only for methylation data obtained from a single plate (plate number four). This plate contains methylation data for year 2008 and includes results for 24 males with alcohol use disorder and 55 males without the disorder. Therefore, final sample size is smaller (24 + 55) and methylation data is not longitudinal as initially planned.

2.2. Control variables

Besides alcohol abuse, other characteristics can influence methylation. These characteristics are gender, age, and smoking frequency, as well as *MAOA* genotype. Values for these variables are shown in Table 1.

Gender. Only male participants were chosen to reduce the complexity of study. Females have two X-chromosomes and one of them is inactivated for dosage compensation, partly by methylation (Wutz, 2011). *MAOA* gene is localized on the X-chromosome (Bortolato et al, 2008). Therefore, one copy of *MAOA* gene is silenced in females. In addition, *MAOA* methylation could differ in males and females (Domschke et al, 2012). These are the reasons why including females would make the analysis more complex.

Age. It is important to control for age because MAO-A activity may change during lifespan (as cited by Shumay & Fowler, 2010, p. 326). This means that methylation could also change in time. In this study, age is automatically controlled for because participants were measured at similar ages.

Smoking frequency. Smoking can influence *MAOA* methylation (Philibert et al, 2009). The percentage of people who smoked daily or almost daily is larger in the AUD group (56.5% *vs.* 37.8%), and the percentage of nonsmokers is lower (21.7% *vs.* 42.2%). Thus, the sampled groups differ in smoking.

***MAOA* genotype.** The number of repetitive DNA sequences in the promoter region of *MAOA* gene ("*MAOA* genotype") might also influence MAO-A activity, as summarized by Shumay et al. (2012, p. 1151). But the effect of genotype may not be very important, because Shumay et al. did not find a relationship between *MAOA* genotype and MAO-A brain activity. Moreover, the distribution of genotypes is similar between two groups, so this influence is sufficiently controlled for.

In conclusion, participants are similar in most of the important characteristics (age, sex, *MAOA* genotype), but they differ in smoking frequency which needs to be controlled for.

Table 1

Values of control variables for AUD group and control group

Control variable	AUD group	Control group
Number of males, *n* (%)	24 (100.0)	55 (100.0)
Approximate age in 2008	25	25
Smoking frequency in 2008, *n* (%)		
never	5 (21.7)	19 (42.2)
few times	2 (8.7)	4 (8.9)
1-2 times per month	2 (8.7)	2 (4.4)
1-2 times per week	1 (4.4)	3 (6.7)
almost every day	2 (8.7)	3 (6.7)
every day	11 (47.8)	14 (31.1)
MAOA VNTR, *n* (%)		
3	11 (45.8)	23 (41.1)
4	11 (45.8)	33 (58.9)
5	2 (8.3)	0 (0.0)

Note. AUD group = group where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008; *MAOA* VNTR = number of repetitive sequences in *MAOA* promoter region. Smoking frequency data is not available for 11 people in the control group.

2.3. Measures and data screening

Lifetime incidence of alcohol use disorder (AUD). Mini International Neuropsychiatric Interview (MINI) was conducted in 2008 by experienced clinical psychologist to assess whether participants had suffered from alcohol use disorder in their life (for references, see Laas et al., 2014, p. 2).

Stressful life events. Participants reported the number of stressful events in 1998, 2001 and 2008. They were given a list of stressful events and they had to indicate which of these they had experienced. Events included parental death, parental alcoholism, emotional abuse and other adverse occurrences (see Kiive et al., 2014, p. 22). For each person, the number of events was counted. This served as a measure of stress. However, the lists varied across years. They were not made comparable, because stress is not the main focus of this study.

Methylation. Methylation was measured in Würzburg University with bisulfite-method (see Appendix 1). Methylation was measured at 12 DNA sites that are located in the promoter region of *MAOA* gene (see Shumay et al., 2012, p. 1152). These sites are called "CpG sites"[3] and denoted as CpG1, CpG2, ..., CpG12. Numbering of these sites is shifted relative to the study of Shumay and colleagues – CpG1 in this study corresponds to CpG3 in Shumay study, CpG2 corresponds to CpG4, etc. In this article, I report results only for CpG9, CpG10 and average methylation across all twelve CpG sites ("CpGmean"), because these variables were shown to have highest correlations with MAO-A brain activity. For CpG9, Spearman *r* was –0.62, for CpG10, Spearman *r* was –0.62, and for CpGmean, Pearson *r* was –0.61 (Shumay et al., 2012, p. 1153–1154). Methylation is expressed as "the proportion of methylated DNA molecules" (see Table 2).

Data screening. I analyzed descriptive statistics, missing values, outliers and shape and found data to be suitable. Most of the methylation and stress variables were not normally distributed (see Appendix 2).

Table 2

Summary of the measures used in this study

Measure	Possible values	Description
Lifetime incidence of alcohol use disorder	1, 0	Presence of alcohol use disorder during life (1 = *yes*, 0 = *no*), assessed with MINI interview in 2008.
Stressful life events	0, 1, 2, …	Number of stressful life events, self-reported in 1998, 2001 and 2008. Participants were given a list of adverse events and had to indicate which of these they had experienced.
Methylation	0 … 1	Methylation of *MAOA* promoter region, measured at 12 DNA sites ("CpG sites"). Takes values between 0 and 1 which show the proportion of methylated DNA molecules at a given site. For example, if CpG9 = 0.33, then 33% of DNA molecules were methylated at site CpG9. This study reports the results for CpG9, CpG10 and average methylation across all 12 CpG sites (CpGmean).

[3] Only the cytosines (C) that are followed by guanine (G) can be methylated. For this reason, methylated sites are called "CpG" sites, where *p* refers to the phospodiester chemical bond which connects the C and G base pairs. (Lodish et al., 2013, p. 18, 296, 327; Klose & Bird, 2006)

2.4. Statistical tests

Choice of tests. I considered both parametric and nonparametric tests and came to the conclusion that nonparametric tests are the safest choice (see Appendix 3 for details). For the simple two-group comparisons (research questions 1 and 3), I use Wilcoxon rank-sum test[4]. For comparing methylation proportions of two groups while controlling for smoking (research question 2), I use logistic regression. When methylation and smoking are in the same regression model, it is possible to find the effect of methylation while the values of smoking are held constant. In addition, even though my hypotheses are directional, I will only calculate two-sided P-values. This assures that important results are not missed, when an opposite difference is found (Agresti & Franklin, 2014, p. 418).

Statistical software. I performed all statistical tests with the free version of SAS (SAS Institute, 2014).

Effect sizes. American Psychological Association recommends the reporting of effect sizes. Effect sizes give additional information. For example, when effects are large but P-values are nonsignificant, further research with higher power may be warranted. (Fritz, Morris, & Richler, 2012, p. 1) For Wilcoxon rank-sum test, I report the rank correlation coefficient *r*. For logistic regression, I mainly report the probability of belonging to the AUD group at minimum and maximum methylation values. When reporting these effect sizes, I will describe their conventional interpretation. However, these are only a guideline – whether an effect size should be considered "small" or "large" depends on the research goals (Fritz et al., 2012, p. 15). For example, Rosnow and Rosenthal (as cited by Fritz et al., 2012) say that if a new procedure has a small effect size for preventing heart attacks, it could still save lives.

Statistical power. It is possible that the results of this study are not statistically significant, even though a significant difference exists in the population. Therefore, power calculations are useful for interpreting the study's results. Unfortunately, power calculations for Wilcoxon rank-sum test (Zhao, Rahardja, & Qu, 2008) and logistic regression (Agresti, 2007, p. 160) seem complicated and I found no free and simple software for that purpose. However, power calculations for t-test are simpler. In this study, where sample sizes are 24 and 55, power of independent samples t-test would be approximately 60% for detecting medium effect size (Aron, Coups, & Aron, 2013a, p. 294, 299). This suggests that power may be less than optimal in this study, even though I did not do precise calculations.

Multiple comparisons. If one would perform many statistical tests at the same time for variables that are independent of each other, it is possible that one of these tests turns out significant just by chance (Bender & Lange, 2001). In this study, I am performing three tests at the same time (comparing CpG9, CpG10 and average methylation). As the number of tests is small and these variables are correlated (Spearman *r* is between 0.35 and 0.67, $p < 0.01$), adjusting for multiple comparisons does not seem a problem.

[4] Wilcoxon rank-sum test is equivalent to Mann-Whitney U test (Agresti & Franklin, 2014, p. 730).

3. Results

3.1. Is methylation lower in the AUD group?

Nonparametric test P-values. According to Wilcoxon rank-sum test, groups did not differ in methylation. This applied to sites CpG9 and CpG10, as well as average methylation. P-values for these three variables were higher than 0.25. Results are listed in Table 3.

Nonparametric test effect sizes. Even when the differences are not statistically significant, it is useful to check effect sizes (see 2.4). For CpG9 and CpG10, effect size *r* was 0.11 and 0.13, respectively. For average methylation, $r = 0.05$. According to McGrath and Meyer (2006, p. 390), $r = 0.10$ could represent small effect size and $r = 0.24$ medium effect size. Therefore, effect sizes for CpG9 and CpG10 can be considered small.

Additional calculations. When extreme outliers ($z > 2.5$) were deleted for each of the methylation variables, the pattern of results did not change. This was expected, because Wilcoxon test is less sensitive to outliers (Fay & Proschan, 2010, p. 25). Scatterplots of methylation proportions (Appendix 4), as well as means and medians (Appendix 2) also confirmed that one group did not have clearly higher methylation proportions than the other group. However, medians were slightly higher in the AUD group (CpG9: 0.16 *vs.* 0.14; CpG10: 0.16 *vs.* 0.15; CpGmean: 0.22 *vs.* 0.21).

Table 3

Comparison of methylation proportions between AUD group and control group by Wilcoxon rank-sum test

Variable	Mean rank in AUD group	Mean rank in control group	*N* AUD	*N* control	*Z* asymp	*p* asymp	Effect size (*r*)
CpG9	43.85	38.32	24	55	0.98	0.33	0.11
CpG10	44.48	38.05	24	55	1.14	0.25	0.13
CpGmean	41.67	39.27	24	55	0.42	0.67	0.05

Note. CpG9 = methylation at site CpG9 in year 2008; CpGmean = average methylation across all CpG sites in year 2008; AUD group = group where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008; *Z* asymp. = z-test statistic for large-sample Wilcoxon test; *p* asymp = p-value for Wilcoxon rank-sum test based on the z-statistic; effect size (*r*) = correlation between alcohol abuse and methylation (when observations are ranked), a measure of effect size.

3.2. Is methylation lower in the AUD group, when smoking is controlled for?

Controlling for smoking with logistic regression. I performed logistic regression separately for each of the three methylation variables. Firstly, I looked at models where the predicted variable is lifetime incidence of alcohol use disorder (1 = *yes*, 0 = *no*) and predictor variable is methylation. Secondly, I added smoking frequency to the model. This shows whether controlling for smoking improves the predictive power of methylation. I also combined the six categories of smoking variable into three, so that frequencies of people would be higher and results more trustable. The three new categories can be labelled as: 0 = *nonsmokers*, 1 = *intermediate smokers*, 2 = *daily smokers*. After fitting the models, I looked at P-values, effect sizes and model fit statistics. I also checked for the influence of outliers or missing values. See Appendix 5 for details.

Initial results for logistic regression. When smoking was controlled for, methylation at gene sites CpG9 and CpG10 tended to predict alcohol abuse ($p = 0.14$ for both sites). When interaction with smoking was controlled for, average methylation predicted alcohol abuse ($p = 0.02$). Effect of average methylation was largest for nonsmokers – if average methylation changed from lower quartile to upper quartile value, probability of alcohol abuse in the sample increased 26%. The effect was smaller for intermediate smokers (change in probability = 16%) and even smaller and opposite for daily smokers (change = –11%). This suggested that there was an interaction between methylation and smoking and that methylation tended to be higher in the AUD group. In addition, even though results for CpG9 and CpG10 were affected by outliers or missing values, the model for average methylation seemed robust to outliers. See Appendix 5.

The inadequacy of logistic regression results. As mentioned above, the effect of average methylation was large in the nonsmokers' group. However, the number of people with AUD was not large in that group ($N = 5$). Only later it occurred to me that this small group could be responsible for all of the findings. In order to test this, I deleted 3 people (who use alcohol harmfully) from the nonsmokers' group. After that, P-values for CpG9, CpG10 and average methylation turned out statistically insignificant – they were not significant (or near significance) in models where methylation was the only predictor, where smoking was included, or where interaction with smoking was included. Additional evidence also suggested that these three people in the nonsmokers' group had large influence.[5] I did not detect these three people in the outlier analysis, because their z-scores were not high and they were not clearly visible in the diagnostic plots provided by statistical software. This suggested two things. First, the difference between AUD group and control group was only seen for nonsmokers (but not for intermediate smokers or daily smokers, as the interaction model suggested). Second, this difference was strong, but it was less trustable due to very small sample size. Figure 1 shows methylation proportions for nonsmokers.

[5] In addition, I recategorized the smoking variable into two categories (daily smokers *vs.* all others), so that the number of people in both categories is larger. The scores of these 3 people still determined the effects of methylation in regression models that include smoking. I also looked at the intermediate smokers' group and daily smokers' group separately. If the interaction model (that showed a large effect for methylation) would be correct, the effect of methylation on alcohol abuse should be visible in these groups. I found no effect, which again suggests that the effect of the nonsmokers' group determined the models.

Association between methylation and alcohol abuse in the nonsmokers' group. To quantify the difference between AUD group and control group in the nonsmokers' subsample, I performed Wilcoxon rank-sum test. Because sample size is small, I chose the exact-distribution version of this test (Agresti & Franklin, 2014, p. 731). According to Wilcoxon test, methylation was higher in the AUD group. This applied to site CpG10 ($p = 0.05$) and average methylation ($p = 0.01$). In order to assess effect size, I calculated the proportion of better responses for AUD group (Agresti & Franklin, 2014, p. 735). With small sample, this measure of effect size could be more appropriate than correlation. Proportion of better responses in the AUD group was 0.86 for average methylation. In other words, when the observations of AUD group and control group were paired in all possible combinations, AUD group had a higher result in 86% of the pairs. If there would have been no effect, this percentage would have been 50. This means that effect size for both average methylation (0.86) and CpG10 (0.80) was considerable. Results are shown in Table 4.

Table 4

Comparison of methylation proportions between AUD group and control group when only nonsmokers are included in the sample

Variable	Mean rank in AUD group	Mean rank in control group	*N* AUD	*N* control	*p* exact	Effect size (Proportion of better responses)
CpG9	15.40	11.74	5	19	0.32	0.66
CpG10	18.00	11.05	5	19	0.05	0.80
CpGmean	19.40	10.68	5	19	0.01	0.86

Note. CpG9 = methylation at site CpG9 in year 2008; CpGmean = average methylation across all observed gene sites in 2008; AUD group = group where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008; *p* exact = p-value for Wilcoxon rank-sum test based on the exact distribution.

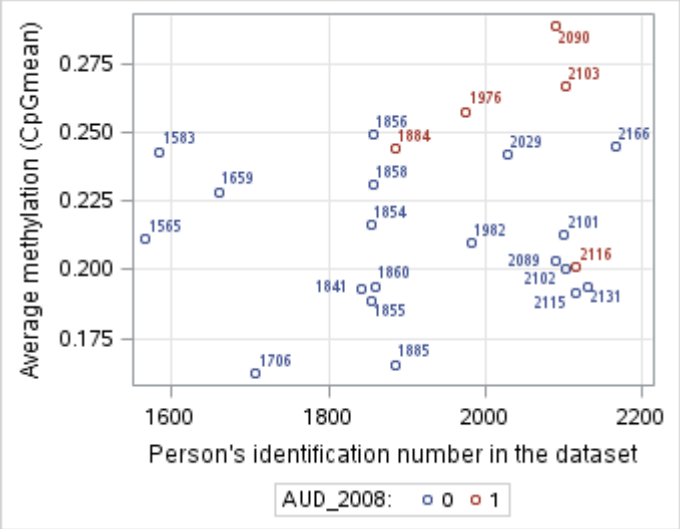

Figure 1. Methylation proportions for nonsmokers in the AUD group (red) and control group (blue). X-axis shows the identification number of each person. Y-axis shows the average methylation proportion calculated across observed gene sites. AUD group = the group of people where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008.

3.3. Has there been more stress in the AUD group?

Nonparametric tests. According to Wilcoxon rank-sum test, the number of stressful life events recorded in 2001 was higher in the AUD group compared to the control group ($p = 0.03$, $r = 0.25$). Effect size r could be considered medium. However, there were no significant differences in the number of stressful events recorded in 1998 and 2008. Results are listed in Table 5.

Checks for outliers. If outliers ($z > 2.5$) were deleted for each of the stress variables (two outliers for stress 1998 and 2001, one outlier for stress 2008), the pattern of results did not change. However, P-value for stress 2001 was somewhat lower ($p = 0.01$).

Additional calculations. In order to better interpret the results, I made the following calculation (as described by Agresti & Franklin, 2014, p. 466). The number of people who had experienced at least 3 stressful life events by year 2001 was 31.5% higher in the AUD group compared to the control group. 95% confidence limits of this difference were [8.7%, 54.4%]. This comes from the fact that 14 people out of 24 (58.3%) in the AUD group had experienced at least 3 stressful events, compared to 15 people out of 56 (26.8%) in the control group.

Table 5

Comparing the number of stressful life events between AUD group and control group by Wilcoxon rank-sum test

Variable	Mean rank in AUD group	Mean rank in control group	*N* AUD	*N* control	*Z* asymp	*p* asymp	Effect size (*r*)
Stress 1998	44.19	38.92	24	56	0.95	0.34	0.11
Stress 2001	48.13	35.89	23	55	2.22	0.03	0.25
Stress 2008	40.63	40.45	24	56	0.03	0.98	0.00

Note. Stress 1998 = number of stressful life events as reported in 1998; AUD group = group where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008; *Z* asymp. = z-test statistic for large-sample Wilcoxon test; *p* asymp = p-value for Wilcoxon rank-sum test based on the z-statistic; effect size (*r*) = correlation between alcohol abuse and stress (when observations are ranked), a measure of effect size.

4. Discussion

4.1. Limitations of this study

First, sample size is small ($N = 24 + 55$), especially when nonsmokers are analysed separately ($N = 5 + 19$). Therefore, when all people are analyzed together, the statistical power may not be optimal for detecting a difference. When only nonsmokers are analyzed, the sample may not be representative of nonsmokers with alcohol use disorders.

Second, incidence of alcohol use disorder is assumed to be a binary variable – a person can either have a disorder or not. But there could be different degrees of alcohol abuse. For example, among those people who are not diagnosed with alcohol use disorder, there could be many who use alcohol in an unhealthy way, but who do not reach the threshold of diagnosis. If this is so, then using a discrete measure (yes/no) can lead to loss of information – people who use alcohol in a rather harmful way may be classified in the same category with people who do not use it at all (Markon, Chmielewski, & Miller, 2011, p. 869; Aron et al., 2013a, p. 398). This could be true in the current study, as alcohol use in the control group is high (48.2% of people in the control group used alcohol every week).

Third, this study supposes that the methylation of *MAOA* gene in white blood cells is an indicator for the activity of MAO-A inside the brain. Indeed, Shumay et al. (2012) found a correlation in the order of 0.60. According to one interpretation, $r = 0.60$ means that 36% of the variability in MAO-A brain activity can be explained by methylation (Agresti & Franklin, 2014, p. 594). This means that the two groups could differ in monoamine oxidase brain activity, but this difference may not be reflected in methylation.

Fourth, groups are not equal in smoking, which can also affect methylation. Smoking can be statistically controlled, but statistical control presupposes that the effect of each smoking frequency is similar to different people. In reality, it is probably not. This can make the results less precise. Nevertheless, statistical control might often be the best choice. It may be hard to get a sample of people who use alcohol harmfully but who do not smoke, because alcohol abuse and smoking often co-occur (as cited by Engle, McIntosh, & Drenan, 2014, p. 13). In addition, groups were not randomly assigned. But this is inevitable for case-control studies.

Nevertheless, this study also has strengths. The groups *are* equal in several important control variables, the sample size probably allows to detect large effect sizes, methylation is considerably correlated to MAO-A brain activity, and results can be generalized to humans (this would be harder if it was an animal study).

4.2. Were the hypotheses confirmed?

First, this study shows that methylation could be higher in the AUD group, but only when nonsmokers are analyzed separately. Is this result reliable? Very small sample size means it is not. Regardless of the significant P-value, AUD group had only five people which means that findings could be due to chance. On the other hand, there are reasons for assuming that methylation is associated with alcohol abuse in the nonsmokers' group, but not in the smokers' group (see 4.5). Therefore, this study suggests that methylation could be higher, even though evidence is weak. Future studies with larger samples are needed.

Second, no differences between AUD group and control group were found in the overall sample. More precisely, there was no difference in the smokers' group. Can it be concluded that there is no such difference in the population? Considering the limitations of this study (4.1), the answer is "probably not". However, because sample size could be sufficient for detecting large effect sizes, this study suggest that there may not be a large difference in population between these two groups.

Third, groups differ in the number of stressful life events. It is thus possible that methylation mediates the relationship between stress and alcohol abuse, although this relationship might apply only to nonsmokers. In addition, the difference between groups was found only for the number of stressful events reported in 2001, but not in 1998 or 2008. The reason for this is unclear, because the lists of stressful events varied across years. I did not investigate this more, as the question of stress was not the main focus of the study.

4.3. Benefits of studying *MAOA* methylation

In section 1.3, I listed three potential benefits of studying methylation. Does this study confirm the benefits?

On one hand, this study does not support the idea that methylation could become a diagnostic marker for alcohol abuse. If methylation would be used to assess the risk of alcohol dependency, it would need to clearly differentiate people who abuse alcohol from people who do not. As methylation can be influenced by smoking, it is not a reliable marker, especially because alcohol use is often accompanied by smoking (as cited by Engle, McIntosh, & Drenan, 2014, p. 13).

On the other hand, this study supports the ideas that MAO-A could play a role in alcohol abuse and that stress might mediate this relationship. Therefore, the results suggest that studying methylation can be useful, even though methylation might not become a diagnostic marker.

4.4. Relationship between methylation and alcohol abuse

What could be the reason for higher methylation in the AUD group? And more generally, how could methylation influence alcohol abuse?

A simple hypothesis might look like this. It is believed that ethanol stimulates dopamine neurons in ventral tegmental area, which leads to the release of dopamine in nucleus accumbens. This release could underlie the rewarding and reinforcing effects of alcohol (Morikawa & Morrisett, 2010). On the other hand, higher methylation is associated with lower MAO-A brain activity (Shumay et al., 2012). Thus, if MAO-A activity is lower, breakdown of dopamine might be slower and people might be more sensitive to the rewarding and reinforcing effects of alcohol.

In addition, norepinephrine could stimulate dopamine release in nucleus accumbens through direct and indirect pathways. (Weinshenker & Schroeder, 2007, p. 1439 –1440) If MAO-A activity is lower, the concentration of norepinephrine may be higher. Higher concentration of norepinephrine could lead to higher concentration of dopamine, which would again make people more sensitive to ethanol.

This simple hypothesis can hold even if the actual mechanisms of ethanol on dopamine are complex (the complexity was stressed by Wu, Gao & Taylor, 2014, p. 311–312). For example, Morikawa and Morrisett (2010) describe how ethanol can influence dopamine neurons directly and indirectly. In direct influence, ethanol affects the intrinsic firing rhythm of dopamine neurons through various ion channels. In indirect influence, ethanol influences other neurotransmitters, which in turn influence dopamine release. But regardless of how dopamine is released, MAO-A could still influence its breakdown.

On the other hand, this hypothesis may be too simple, because MAO-A is not the only enzyme involved in the breakdown of dopamine and norepinephrine (Bortolato et al., 2008, p. 1528–1529). In addition, MAO-A is located inside neurons as well as glial cells (Youdim, Edmondson & Tipton, 2006). This means that there could be different pathways by which MAO-A influences dopamine. Moreover, various other factors can influence alcohol use. For example, Zhang et al. (2013, p. 395) found that 1710 gene sites (not genes) were differently methylated in people with alcohol dependency. And these are only biological factors. There could also be psychological and social factors.

Also, this hypothesis presupposes that methylation increases the risk of alcohol abuse. But alcohol use itself (like smoking) could affect methylation. This study does not give information about the direction of influence, because data is not longitudinal.

4.5. Interaction between smoking and methylation

The idea that methylation is associated with alcohol abuse, but only for nonsmokers, seems to fit with the results of a recent study. Engle, McIntosh and Drenan (2015, p. 17) found that low concentrations of nicotine and ethanol, when applied together, evoked electrical potentials from dopamine neurons, but these substances did not have such effect when applied separately in their small concentrations. This means that the overall effect of nicotine and ethanol can be much stronger when these substances are used together. In fact, authors estimate that just one cigarette and one drink could be enough to induce plastic changes in dopamine neurons. Therefore, even if lower methylation makes people less prone to ethanol addiction (see 4.4), it may not be enough to protect them from the co-exposure of these two substances. When nicotine and ethanol are used together, the effect may be so strong that it overrides the effect of methylation.

4.6. Ideas for future studies

There are various ways for studying the role of MAO-A in alcohol abuse. For example:

- A study similar to this one, where sample size is larger, alcohol use frequency in the control group is smaller, and methylation data is longitudinal (which is possible in the ECPBHS sample).
- A neuroimaging study with humans where the activity of MAO-A is measured directly in the brain for cases and controls. The measurement protocol could be similar to Shumay et al. (2012). In addition, it might be interesting to do an experiment where the brain activity of MAO-A is measured before and after ethanol consumption. These studies are expensive, but can give strong evidence.
- A biological study that measures the effect of MAO-A inhibitors on the electrical potentials evoked from dopamine neurons. The protocol could be similar to Engle et al. (2015).

Biological mechanisms of alcohol abuse could also be studied more generally with new technologies. For example, it would be interesting to analyze microRNA-s obtained from human blood. MicroRNA-s are molecules that are suggested to be master regulators of protein expression and they could be novel diagnostic biomarkers for human disease (as referenced by Gorini, Nunez, & Mayfield, 2013). Indeed, Gorini et al. (2013) found that microRNA levels were different in ethanol dependent mice.

4.7. Conclusion

In summary, this study shows that methylation could be related to alcohol abuse, but only in the nonsmokers' group – perhaps a novel finding. It also leaves open the possibility that, for nonsmokers, methylation mediates the relationship between stress and alcohol abuse. However, due to small sample size and other limitations, these findings should be considered as initial and in need of replication.

Appendices

Appendix 1. How was methylation measured?

Methylation data was obtained similarly to Shumay and colleagues (2012, p. 1156). The research team led by Shumay used the following steps.

1. DNA was isolated from white blood cells in venous blood.
2. Isolated DNA was treated with a chemical called sodium bisulfite, which converts unmethylated cytosines to uracil. See Figure 2.
3. Polymerase chain reaction (PCR) was carried out with bisulfite-treated DNA. PCR enables to "take out" the region of interest from the DNA so that this region can be further analysed. When PCR is complete, researchers have a collection of DNA molecules which contain only the region of interest. The region of interest in this study is the promoter region of monoamine oxidase A (MAO-A) gene.
4. PCR products were incorporated into a circular DNA molecule (plasmid). These circular molecules were inserted into bacterial cells. This step is important for getting enough DNA for analysis. Even though the PCR reaction "takes out" the region of interest, the number of DNA molecules is too small for further study. But when the region of interest is inserted into bacteria, it is multiplied. When bacteria grow and divide, they also replicate the DNA that is inserted into them.
5. As a final step, *MAOA* DNA was extracted from bacterial cells and its sequence was determined in order to know which gene sites were methylated.

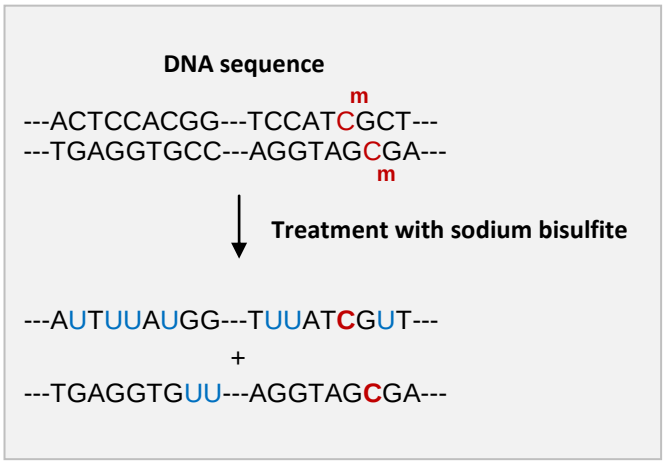

Figure 2. When DNA is treated with sodium bisulfite, the chemical converts unmethylated cytosines (C) into uracil (U). Later, when the DNA sequence is determined, the number of uracils indicates the number of unmethylated sites. Figure is adapted from Wikipedia (2007).

Appendix 2. Data screening, descriptive statistics and normality tests

Data screening procedure. To see whether data is suitable for analysis, I used the following procedure, which I adapted from one statistics textbook (Aron, Coups & Aron, 2013b, p. 19–23):

- Data table – are there any unusual observations or unusual patterns visible in the data table?
- Descriptive statistics – are all the minimums, maximums, means and standard deviations within theoretically possible boundaries?
- Missing values – are there many missing values? If so, did they arise randomly?
- Outliers – do some people have extremely high scores (absolute value of z-score > 3 or > 2.5)?
- Normal distributions – which of the variables are approximately normally distributed?
- Shape – which of the variables have approximately the same shape?

Descriptive statistics. In general, all descriptive statistics were in theoretically possible boundaries and none of them seemed extreme at first glance. Descriptive statistics are listed in Table 6.

Missing values. The number of missing values seemed acceptable too. In the AUD group, there were no missing values in the main methylation variables and no more than 2 missing values in each of the other variables. In control group, there was only 1 missing value in each of the main methylation variables and no more than 2 missing values in the each of the other variables. Smoking frequency variable was the only exception – there were 11 missing values in the control group. There did not seem to be a systematic reason for this, because these people reported their alcohol frequency (they did not choose to "hide it").

Outliers. There were 7 outliers in the three main methylation variables ($z > 2.5$), 5 in the control group. These outliers seemed to be randomly dispersed over the data table. However, one person had 8 extreme observations across all methylation variables. It is unlikely to be measurement error, because all blood samples were analyzed together. In addition, if methylation proportions in the population were normally distributed, the probability of having outliers (defined by $z > 2.5$ or $z < –2.5$) would be approximately 1.2%. As the total sample size was 79, there should have been about 1 outlier in the sample (0.012*79). This suggested, in addition to other evidence below, that observations in the population may not be normally distributed.

Normal distributions. According to Shapiro-Wilk test, none of the variables were normally distributed in both groups. Focusing on methylation variables, quantile-quantile plots and histograms (not shown) confirmed that methylation was not normally distributed; the reason seemed to be both in skewness and kurtosis (see table 7).

Could the data be corrected to make it normally distributed? If six people were deleted from the dataset who had extreme observations ($z > 2.5$ or $z < –2.5$), all of the main methylation variables could be considered normally distributed. However, deletions did not seem good for two reasons. First, deletions would have

meant a considerable loss of data (about 7%). Second, it is likely that methylation variables are not normally distributed in the population, because methylation can take only positive values between 0 and 1 and the average for most gene sites is not around 0.5 – both in this data and in the data of Shumay et al. (2012, p. 1157). This can result in skew due to restricted range of values on one side of the mean. Data transformation did not seem good either. First, it would have distorted the original data units and made the results harder to interpret. Second, logarithm and square-root transformations did not result in the normality for all variables of interest.

Shape. None of the variables had similar shape in terms of skewness, except for smoking.

Table 6

Descriptive statistics for control variables and comparison variables

Variable	Possible values	Mean	Std Dev	Min	Max	Median	Lower Quartile	Upper Quartile	*N*	*N* Miss
AUD group										
Smoking_2008	1, 2, 3, 4, 5, 6	4.13	2.14	1	6	5	2	6	23	1
Stress_1998	0, 1, 2, …	1.83	1.24	0	4	2	1	3	24	0
Stress_2001	0, 1, 2, …	3.00	2.17	0	7	3	1	5	23	1
Stress_2008	0, 1, 2, …	3.92	2.45	0	9	4	2	6	24	0
*MAOA*_VNTR	2, 3, 3.5, 4, 5	3.63	0.65	3	5	4	3	4	24	0
CpG9	0 … 1	0.16	0.07	0.07	0.40	0.16	0.11	0.18	24	0
CpG10	0 … 1	0.18	0.07	0.08	0.35	0.16	0.14	0.22	24	0
CpGmean	0 … 1	0.22	0.04	0.17	0.35	0.22	0.19	0.24	24	0
Control group										
Smoking_2008	1, 2, 3, 4, 5, 6	3.20	2.23	1	6	2	1	6	45	11
Stress_1998	0, 1, 2, …	1.57	1.29	0	5	1	1	2	56	0
Stress_2001	0, 1, 2, …	1.80	1.65	0	6	1	1	3	55	1
Stress_2008	0, 1, 2, …	3.98	2.71	0	11	4	2	6	56	0
*MAOA*_VNTR	2, 3, 3.5, 4, 5	3.59	0.50	3	4	4	3	4	56	0
CpG9	0 … 1	0.14	0.04	0.05	0.26	0.14	0.11	0.16	55	1
CpG10	0 … 1	0.16	0.06	0.05	0.44	0.15	0.13	0.18	55	1
CpGmean	0 … 1	0.21	0.03	0.16	0.30	0.21	0.19	0.24	55	1

Note. AUD group = group where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008; smoking_2008 = smoking frequency in last 12 months in 2008 (1 = *never*; 2 = *few times*; 3 = *one or two times per month*; 4 = *one or two times per week*; 5 = *almost every day*; 6 = *every day*); stress_1998 = number of stressful life events reported in 1998; *MAOA*_VNTR = number of repetitive sequences in the promoter region of *MAOA* gene; CpG9 = methylation at site CpG9 in year 2008; CpGmean = average methylation across all CpG sites in year 2008; Std Dev = standard deviation; min = minimum value; N miss = number of missing values.

Table 7

Shape characteristics for control variables and comparison variables

Variable	Skew	Kurt	SE skew	Z skew	SE kurt	Z kurt	Shapiro-Wilk
AUD group							
Smoking_2008	-0.52	-1.57	0.48	-1.08	0.93	-1.68	<0.0001
Stress_1998	0.19	-1.16	0.47	0.41	0.92	-1.26	0.01
Stress_2001	0.35	-1.13	0.48	0.72	0.93	-1.20	0.02
Stress_2008	0.42	-0.52	0.47	0.88	0.92	-0.56	0.27
*MAOA*_VNTR	0.54	-0.52	0.47	1.15	0.92	-0.57	<0.0001
CpG9	1.86	6.00	0.47	3.94	0.92	6.54	0.00
CpG10	0.98	0.46	0.47	2.07	0.92	0.50	0.04
CpGmean	1.15	1.35	0.47	2.44	0.92	1.48	0.04
Control group							
Smoking_2008	0.25	-1.80	0.35	0.71	0.69	-2.60	<0.0001
Stress_1998	0.81	0.34	0.32	2.53	0.63	0.54	0.00
Stress_2001	0.85	-0.13	0.32	2.63	0.63	-0.20	<0.0001
Stress_2008	0.64	-0.03	0.32	2.01	0.63	-0.05	0.01
*MAOA*_VNTR	-0.37	-1.93	0.32	-1.17	0.63	-3.07	<0.0001
CpG9	0.40	0.52	0.32	1.24	0.63	0.82	0.32
CpG10	1.96	6.29	0.32	6.08	0.63	9.93	<0.0001
CpGmean	0.29	-0.05	0.32	0.91	0.63	-0.08	0.43

Note. Descriptions for variables are given in the previous table. Skew = skewness; kurt = kurtosis; SE skew = standard error of skewness; Z skew = z-score for obtaining a sample with such skewness from a population where skewness is 0; Shapiro-Wilk = P-value for Shapiro-Wilk normality test.

Appendix 3. Statistical tests considered for this research project

Table 8

Statistical tests considered for this research project: tests are grouped according to research questions

Research question	Statistical test	Assumptions	Assumptions fulfilled?
1. Is methylation lower in the AUD group? Has there been more stress in the AUD group?	1.1. Wilcoxon rank-sum test	(1) Response variable is measured at least on ordinal scale; (2) independent random samples. (Agresti & Franklin, 2014, p. 728)	YES – Assumption (1) is met. Assumption (2) is met, but more weakly due to nonrandom sample.
	1.2. Hodges-Lehmann estimate for the difference between medians	(1) Response variable is measured at least on ordinal scale; (2) independent random samples; (3) population distributions have the same shape. (Agresti & Franklin, 2014, p. 730)	NO – Assumption (3) is not met.
	1.3. Independent samples t-test	(1) Response variable is quantitative; (2) independent random samples; (3) approximately normal population distribution for each group or sample size larger than 30 in each group. (Agresti & Franklin, 2014, p. 481)	NO – Assumption (3) is not met. Distributions are not normal and sample size in the smaller group is 24.
2. Is methylation lower in the AUD group, when smoking is controlled for?	2.1. Logistic regression	No assumptions about the distribution, variance or relationship between predictor variables (Tabachnick & Fidell, 2001, p. 517). The response variable is a binary category (e.g. 0 *vs.* 1), so it does not have to be normally distributed (Agresti, 2007, p. 67). However, it is good if the following criteria are met: (1) Logit transform of the response variable is linearly related to predictors; (2) Absence of outliers – cases who are very poorly predicted by the model; (3) Independence of errors – all cases are independent; (Tabachnick & Fidell, 2001, p. 522-523); (4) At least 10 observations in each predicted category for every predictor; (5) Absence of high correlations between predictors (multicollinearity); (Agresti, 2007, p. 138).	YES. In addition, criteria (3) and (4) are met – cases and controls are not matched one-on-one; and the model can accommodate at least 2 predictors (methylation and smoking) because there are at least 24 people in the smaller group. Fulfilment of criteria (2) and (5) will be evident from the results. Outliers will be seen from the diagnostic plots (SAS Institute, 2010b , p. 3996) and multicollinearity can be detected when the overall P-value for the model is small, but individual effects are not significant (Agresti, 2007:139). I will not test linearity (1) separately. I assume it is indicated by the fit of the model.
	2.2. General linear model (ANCOVA)	(1) Relationship between predictors and response variables is linear. (2) Residuals are normally distributed at all combinations of the predictor variables. (3) Residuals have the same variance at all combinations of the predictor variables. (Carey, 2004, p.13–14)	MAYBE – methylation variables are not normally distributed in both groups which also means that their residuals are not normally distributed at the two levels of the grouping variable (1 vs. 0). However, I did not test if residuals are normally distributed when smoking is included in the model, and if they have same variance. It would have taken considerable time to learn to use general linear model (in addition to logistic regression). This would have taken more time than planned for this project. I chose logistic regression, because it was safe.

Appendix 4. Scatterplots for methylation proportions

Figure 3. Scatterplots of methylation proportions. Y-axis shows the methylation proportions for sites CpG9, CpG10 and average methylation. X-axis shows the row number of participants. People in the AUD group are marked with red circles. AUD group is the group where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008.

Appendix 5. Logistic regression analysis

Regression models. I performed logistic regression separately for each of the three methylation variables. Firstly, I looked at models where the predicted variable is lifetime incidence of AUD (1 = *yes*, 0 = *no*) and predictor variable is methylation. Secondly, I added smoking frequency to the model. This shows whether controlling for smoking improves the predictive power of methylation. Smoking frequency was missing for 11 people, so I excluded them. I also excluded them from the models that contain methylation as the only predictor, so that these models would be comparable to models that contain both methylation and smoking.

Combining the categories of smoking. In the original data, smoking variable has six categories that represent different degrees of smoking. However, the number of people in most of these categories is very small (see Table 1). I combined the six categories into three, so that frequencies of people would be higher and results more trustable.[6] The three new categories can be labelled as: 0 = *nonsmokers*, 1 = *intermediate smokers*, 2 = *daily smokers*. I treated this new smoking variable as a rank-order variable, having confirmed that logistic regression allows rank-order predictors. (Agresti, 2007, p. 118) I also tried other ways of categorizing and concluded that the three-category choice is appropriate.

P-values and regression coefficients for methylation. Methylation did not predict group membership in a statistically significant way when smoking was controlled for. However, when compared to simpler models that did not contain smoking, P-values for methylation were smaller and standardized regression coefficients were larger for both CpG9 and average methylation. This suggested that controlling for smoking improves the predictive power of methylation. In addition, P-values for CpG9 and CpG10 were not far from 0.05 level of significance ($p = 0.14$ for both sites). Positive regression coefficients for these sites showed that methylation tends to be higher in the AUD group. Table 9 shows these results.

Effect size for methylation. I calculated the probabilities of belonging to the AUD group at minimum and maximum methylation values. The difference between these two probabilities shows effect size. For example, if methylation values change from minimum to maximum, but the probability of alcohol use disorder changes very little, methylation does not have a large effect. However, because minimums and maximums are sensitive to outliers, it is better to use lower quartile (LQ) and upper quartile (UQ). (Agresti, 2007, p. 121) In this calculation, smoking should be fixed at its ordinary value. I chose "0" ("nonsmokers") for the ordinary value, because nonsmokers are most common in the control group.[7] When smoking was fixed at value 0 and methylation increased from LQ to UQ, the probability of belonging to the AUD group increased approximately 6%. For example, at site CpG9, probability increased from 0.15 to 0.21. This effect seemed small, but noteworthy, as methylation changed only over the middle 50% range of its values. But confidence intervals were wide (see 4.1). Table 10 shows the results.

[6] In the original data, smoking has six categories (1 = *never*; 2 = *few times*; 3 = *one or two times per month*; 4 = *one or two times per week*; 5 = *almost every day*; 6 = *every day*). I combined these into three (0 = *never*, 1 = *few times, one or two times per month, one or two times per week*; 2 = *almost every day, every day*). In this case, frequency of people in each category of both groups is at least 5.

[7] In the control group, 42.2% of people never smoked in 2008. They belong to category "0" in the new smoking variable. In comparison, only about 20.0% of people belong to category "1". I chose control group as the reference point, because people who do not use alcohol harmfully are probably much more common the population than people who use alcohol harmfully.

Effect size for the whole model. I also calculated effect size measures for regression models which contain methylation and smoking. Concordance index *c* was between 0.63 and 0.66 and Nagelkerke R^2 was between 0.06 and 0.08.[8] However, these measures describe the whole model, not methylation in particular.

Interactions. There was no interaction between smoking and CpG9 or CpG10. However, smoking interacted with average methylation ($p_{\text{interaction}} = 0.03$, $\beta_{\text{interaction}} = -25.94$). In this model, the effect of methylation was statistically significant ($p = 0.02$). The effect was largest for nonsmokers – if average methylation changed from lower quartile to upper quartile value, probability of alcohol use disorder in the sample increased 26%. Effect was smaller for intermediate smokers (change in probability = 16%) and even smaller and opposite for daily smokers (change = –11%). In addition, effect size of the whole model was considerable ($c = 0.72$, $R^2_{\text{Nagelk.}} = 0.20$). This interaction model is included in Table 9 and shown in Figure 4.

Checks for outliers and missing values. First, I identified extreme observations by looking at z-scores ($z > 2.5$ or $z > 2.0$). I explored the effect of deleting these observations. Second, I looked at diagnostic plots (SAS Institute, 2010b, p. 3996) and identified observations that have large effects on the model. Models for average methylation and CpG10 seemed quite immune to outliers, while the model for CpG9 was more sensitive and thus less reliable. In addition, results for CpG10 were also less trustworthy. The model that contained CpG10 as the only predictor was affected by the exclusion of 11 people for whom the smoking values were missing.[9]

Inadequacy of logistic regression models. As mentioned in section 3.2, all of the logistic regression models that contained smoking as a predictor variable were influenced by the deletion of three observations from the sample. Therefore, none of these models was reliable. These three observations were not detectable from z-scores and they did not appear clearly on the diagnostic plots. However, I still presented all the logistic regression results to show the analysis that I carried out. The inadequacy of these models could have been detected more easily, if I would have used the smoking frequency as a coded categorical variable, in addition to using it as a rank-order variable. I can take this into account in future studies.

[8] I considered the following guidelines for interpreting these effect sizes. Concordance index $c = 0.50$ means that the predictive power of the model is not better than random guessing (Agresti, 2007, p. 144) and Nagelkerke R^2 represents the proportion of variance explained (Nagelkerke, 1991). In ordinary regression, according to Cohen, $R^2 = 0.02$ could represent small effect size and $R^2 = 0.13$ could represent medium effect size (as cited in Aron et al, 2013a). However, R^2 analogues for logistic regression have been criticized (e.g. Menard, 2012, p. 20).

[9] When I calculated the model that contains methylation as the only predictor variable, I excluded the scores of 11 people whose smoking values were missing. If these people were not excluded, P-value of CpG10 would be higher ($p = 0.21$ *vs.* 0.14). Excluding these people did not strongly affect models where CpG9 (0.20 *vs.* 0.25) or average methylation (0.35 *vs.* 035) were the only predictors.

Table 9

Logistic regression models where methylation is a predictor of alcohol abuse: the effects of methylation as a single predictor vs. the effects of methylation when smoking is controlled for

	Statistics for methylation				Statistics for the whole model					
Model	*p*	*β*	95% CI *β*	Std. *β*	*p*	*c* index	R^2 Cox & Snell	R^2 Nagelk.	AIC	–2LogL
CpG9	0.25	5.87	[-3.8, 16.69]	0.17	0.23	0.57	0.02	0.03	88.77	84.77
CpG9 + S	0.14	8.07	[-2, 19.83]	0.23	0.07	0.66	0.08	0.11	86.75	80.75
CpG10	0.13	6.53	[-1.7, 15.39]	0.22	0.12	0.59	0.04	0.05	87.77	83.77
CpG10 + S	0.14	6.46	[-1.88, 15.52]	0.22	0.07	0.65	0.08	0.10	86.90	80.90
CpGmean	0.35	6.68	[-7.34, 21.13]	0.13	0.35	0.54	0.01	0.02	89.30	85.30
CpGmean + S	0.23	8.84	[-5.54, 24.17]	0.18	0.11	0.63	0.06	0.09	87.74	81.74
CpGmean + S + CpGmean*S	0.02	42.81	[11.04, 84.51]	0.85	0.02	0.72	0.14	0.20	83.74	75.74

Note. Models are written in shorthand form. For example, "CpG9 + S" is a logistic regression model that predicts the lifetime incidence of alcohol use disorder (*yes*/*no*) using two predictors: methylation at site CpG9 and smoking frequency. All models contain intercept, only the last model contains interaction. Statistics for methylation are the following: *p* = p-value according to Wald test; *β* = regression coefficient; 95% CI *β* = 95% confidence intervals for *β* according to likelihood-ratio method; Std. *β* = standardized regression coefficient. Statistics for the whole model: *p* = p-value according to likelihood-ratio method; *c* index = concordance index; R^2 Nagelk. = Nagelkerke's coefficient of determination; AIC = akaike information criterion, shows how close the fitted values tend to be to true expected values (smaller AIC is better); –2logL = maximized value of likelihood function.

Table 10

Predicted probabilities of alcohol abuse at small and large methylation values: difference between these probabilities reflects the effect size of methylation

Model	Smoking value	LQ methylation	UQ methylation	Probability at LQ	Probability at UQ	Change in probability
CpG9	-	0.11	0.16	0.29	0.35	0.06
CpG9 + S	0	0.11	0.16	0.15	0.21	0.06
CpG10	-	0.13	0.18	0.29	0.36	0.07
CpG10 + S	0	0.13	0.18	0.18	0.23	0.05
CpGmean	-	0.19	0.24	0.30	0.37	0.06
CpGmean + S	0	0.19	0.24	0.17	0.24	0.06
CpGmean + S + CpGmean*S	0	0.19	0.24	0.05	0.32	0.26

Note. Smoking value "0" represents nonsmokers; LQ methylation = lower quartile value for methylation in the control group; probability at LQ = predicted probability of belonging to the AUD group when smoking is at value 0 and methylation is at lower quartile value; change in probability = probability at UQ minus probability at LQ, a measure of effect size; AUD group = the group where lifetime incidence of alcohol use disorder (AUD) was diagnosed in 2008.

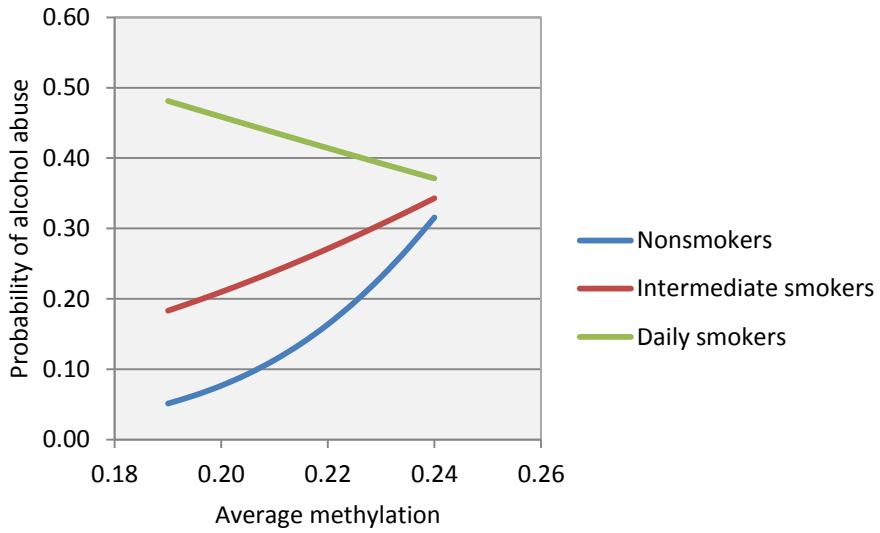

Figure 4. Average methylation predicts alcohol abuse, but its predictive power depends on the frequency of smoking. In the figure, the minimum value of methylation is the lower quartile value of the control group; the maximum value is the upper quartile value. Definitions: probability of alcohol abuse = probability of belonging to the AUD group; average methylation = average methylation proportion across all twelve CpG sites (i.e. average methylation of the studied gene region); nonsmokers = people who reported that they never smoked in 2008; intermediate smokers = people who reported that they smoked a few times, once or twice per month, or once or twice per week in 2008; daily smokers = people who reported that they smoked daily or almost daily in 2008.

References

Agresti, A. (2007). *Categorical data analysis* (2nd ed.). Hoboken, NJ: Wiley.

Agresti, A., & Franklin, C. (2014). *Statistics: The art and science of learning from data* (3rd ed., international ed.). Harlow: Pearson.

Aron, A., Coups, E. J., & Aron, E. N. (2013a). *Statistics for psychology* (6th ed.) Upper Saddle River, NJ: Pearson.

Aron, A., Coups, E. J., & Aron, E. N. (2013b). Applying statistical methods in your own research project. In *Statistics for Psychology* (6th ed., web chapter 2). Upper Saddle River, NJ: Pearson. Retrieved from http://media.pearsoncmg.com/ph/hss/hss_aron_statspsych_6/online_resources/web_chapters/0205888380_W02.pdf

Bender, R., & Lange, S. (2001). Adjusting for multiple testing—when and how? *Journal of Clinical Epidemiology, 54*, 343–349. doi:10.1016/S0895-4356(00)00314-0

Bortolato, M., Chen, K., & Shih, J. C. (2008). Monoamine oxidase inactivation: From pathophysiology to therapeutics. *Advanced Drug Delivery Reviews, 60*, 1527–1533. doi:10.1016/j.addr.2008.06.002

Brenet, F., Moh, M., Funk, P., Feirstein. E., Viale, A. J., Socci, N. D., & Scandura, J. M. (2011). DNA methylation of the first exon is tightly linked to transcriptional silencing. *PLoS ONE 6(1): e14524*. doi:10.1371/journal.pone.0014524

Carey, G. (2004). *QMIN: General linear models (GLM) theory* [PDF document]. Retrieved from http://psych.colorado.edu/~carey/courses/psyc5741/handouts/GLM%20Theory.pdf

Domschke, K., Tidow, N., Kuithan, H., Schwarte, K., Klauke, B., Ambrée, O., Reif, A., Schmidt, H., Arolt, V., Kersting, A., Zwanzger, P., & Deckert, J. (2012). Monoamine oxidase A gene DNA hypomethylation – a risk factor for panic disorder? *International Journal of Neuropsychopharmacology, 15*, 1217–1228. doi:10.1017/S146114571200020X

Engle, S. E., McIntosh, M., & Drenan, R. M. (2015). Nicotine and ethanol cooperate to enhance ventral tegmental area AMPA receptor function via α6-containing nicotinic receptors. *Neuropharmacology, 91*, 13–22. doi:10.1016/j.neuropharm.2014.11.014

Fay, M. P., & Proschan, M. A. (2010). Wilcoxon-Mann-Whitney or t-test? On assumptions for hypothesis tests and multiple interpretations of decision rules. *Statistics Surveys, 4*, 1–39. doi:10.1214/09-SS051

Fritz, C. O., Morris, P. E., & Richler, J. J. (2012) Effect size estimates: current use, calculations, and interpretation. *Journal of Experimental Psychology: General, 141*, 2–18. doi:10.1037/a0024338

Gorini, G., Nunez, Y. O., & Mayfield, R. D. (2013). Integration of miRNA and protein profiling reveals coordinated neuroadaptations in the alcohol-dependent mouse brain. *PLoS ONE 8(12)*: *e82565*. doi:10.1371/journal.pone.0082565

Hester, M. W., Bruford, E. A., Lovering, R. C., Lush, M. J., Wright, M. W., & Povey, S. (2002). Guidelines for human gene nomenclature. *Genomics, 79*, 464–470. doi:10.1006/geno.2002.6748

Kenny, D. A. (2014). Mediation: the four steps. Retrieved from http://davidakenny.net/cm/mediate.htm

Kiive, E., Laas, K., Akkermann, K., Comasco, E., Oreland, L., Veidebaum, T., & Harro, J. (2014). Mitigating aggressiveness through education? The monoamine oxidase A genotype and mental health in general population. *Acta Neuropsychiatrica, 26*, 19 –28. doi:10.1017/neu.2013.34

Klose, R. J., & Bird, A. P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends in Biochemical Sciences, 31*, 89-97. doi:10.1016/j.tibs.2005.12.008

Laas, K., Reif., A., Akkermann, K., Kiive, E., Domschke, K., Lesch, K.-P., Veidebaum, T., & Harro, J. (2014). Neuropeptide S receptor gene variant and environment: contribution to alcohol use disorders and alcohol consumption. *Addiction Biology.* Advance online publication. doi:10.1111/adb.12149

Lodish, H., Berk, A., Kaiser, C. A., Krieger, M., Bretscher, A., Ploegh, H., & Amon, A. (2013). *Molecular Cell Biology* (7th ed., international ed.). New York, NY: W. H. Freeman.

Markon, K. E., Chmielewski, M., & Miller, C. J. (2011). The reliability and validity of discrete and continuous measures of psychopathology: A quantitative review. *Psychological Bulletin, 5,* 856–879. doi:10.1037/a0023678

McGrath, R. E., & Meyer, G. J. (2006). When effect sizes disagree: the case of *r* and *d*. *Psychological Methods, 11,* 386–401. doi:10.1037/1082-989X.11.4.386

Menard, S. (2000). Coefficients of determination for multiple logistic regression analysis. *The American Statistician, 54,* 17-24. doi:10.1080/00031305.2000.10474502

Morikawa, H., & Morrisett, R. A. (2010). Ethanol action on dopaminergic neurons in the ventral tegmental area: interaction with intrinsic ion channels and neurotransmitter inputs. *International Review of Neurobiology, 91,* 235–288. doi:10.1016/S0074-7742(10)91008-8

Nagelkerke, N. J. D. (1991) . A note on a general definition of the coefficient of determination. *Biometrika, 78,* 691-692. doi:10.2307/2337038

Philibert, R. A., Gunter, T. D., Beach, S. R. H., Brody, G. H., & Madan, A. (2008). MAOA methylation is associated with nicotine and alcohol dependence in women. *American Journal of Medical Genetics part B (Neuropsychiatric Genetics), 147B,* 565–570. doi:10.1002/ajmg.b.30778

Philibert, R. A., Beach, S. R. H., Gunter, T. D., Brody, G. H., Madan, A., & Gerrard, M. (2009). The effect of smoking on MAOA promoter methylation in DNA prepared from lymphoblasts and whole blood. *American Journal of Medical Genetics part B (Neuropsychiatric Genetics), 153B,* 619–628. doi:10.1002/ajmg.b.31031

SAS Institute Inc. (2010a). *Base SAS® 9.2 procedures guide: statistical procedures* (3rd ed.). Cary, NC: SAS Institute Inc. Retrieved from http://support.sas.com/documentation/cdl/en/procstat/63104/PDF/default/procstat.pdf

SAS Institute Inc. (2010b). *SAS/STAT® 9.22 User's Guide*. Cary, NC: SAS Institute Inc. Retrieved from http://support.sas.com/documentation/cdl/en/statug/63347/HTML/default/viewer.htm#statug_logistic_sect057.htm

SAS Institute Inc. (2014). SAS OnDemand for Academics. Retrieved from http://www.sas.com/en_us/industry/higher-education/on-demand-for-academics.html

Shumay, E., & Fowler, J. S. (2010). Identification and characterization of putative methylation targets in the MAOA locus using bioinformatic approaches. *Epigenetics, 5,* 325-342. doi:10.4161/epi.5.4.11719

Shumay, E., Logan, J., Volkow, N. D.,& Fowler, J. S. (2012). Evidence that the methylation state of the monoamine oxidase A (*MAOA*) gene predicts brain activity of MAO A enzyme in healthy men. *Epigenetics 7,* 1151—1160. doi:10.4161/epi.21976

Tabachnick, B. G., & Fidell, L. S. (2001). *Using multivariate statistics* (4th ed., international ed.). Needham, MA: Pearson.

Weinshenker, D., & Schroeder, P. (2007). There and Back Again: A Tale of Norepinephrine and Drug Addiction. *Neuropsychopharmacology, 32,* 1433–1451. doi:10.1038/sj.npp.1301263

Wikipedia (2007). Outline of bisulfite conversion of sample sequence of genomic DNA. Retrieved from http://en.wikipedia.org/wiki/Bisulfite_sequencing#mediaviewer/File:Wiki_Bisulfite_sequencing_Figure_1_small.png

Wu, J., Gao, M., & Taylor, D. H. (2014). Neuronal nicotinic acetylcholine receptors are important targets for alcohol reward and dependence. A*cta Pharmacologica Sinica, 35,* 311–315

Wutz, A. (2011). Gene silencing in X-chromosome inactivation: advances in understanding facultative heterochromatin formation. *Nature Reviews Genetics, 12,* 542–553. doi:10.1038/nrg3035

Youdim, M. B. H., Edmondson, D., & Tipton, K. F. (2006). The therapeutic potential of monoamine oxidase inhibitors. *Nature Reviews Neuroscience, 7,* 295–309. doi:10.1038/nrn1883

Zhang, R., Miao, Q., Wang, C., Zhao, R., Li, W., Haile, C. N., Hao, W., & Zhang, X. Y. (2013). Genome-wide DNA methylation analysis in alcohol dependence. *Addiction Biology, 18,* 392–403. doi:10.1111/adb.12037

Zhao, Y. D., Rahardja, D., & Qu, Y. (2008). Sample size calculation for theWilcoxon–Mann–Whitney test adjusting for ties. *Statistics in Medicine, 27,* 462–468. doi:10.1002/sim.2912

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