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MAOA gene methylation in association with alcohol use disorder

Research project

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Running head: MAOA methylation and AUD

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Abstract

Monoamine oxidase A (MAO-A) is a mithocondrial enzyme, that is linked to alcohol use disorder (AUD). One agent involved in MAO-A expression, is *MAOA* gene methylation, which can be measured from white blood cells. Measures of *MAOA* methylation were taken from 12 individual sites (CpG sites). Male subjects were evaluated based on their lifetime incidence of AUD (N_{AUD} = 42, $N_{non\text{-}AUD}$ = 78), smoking, type of alcohol consumed as well as its frequency – analysis was conducted in three different age groups of 15, 18 and 25 years. At 25 years of age, CpG sites 3, 5, 7, and 11 showed statistically lower *MAOA* methylation in case of AUD, CpG11 being seemingly the most sensitive, becaused in this case the association persisted from ages 18 to 25. From AUD-sensitive sites, smoking affected significantly only CpG11 methylation (at age 25). Smoking and light alcohol consumption had an interaction at CpG8 (at age 25), when AUD was not accounted for. When looking for *MAOA* genotype and AUD interactions, CpG sites 3 and 9 at 25 years of age proved to be significant. *MAOA* methylation was usually lower in the case of AUD and number of significant associations increased with age.

Keywords. monoamine oxidase A, *MAOA*, DNA methylation, *MAOA* methylation, alcohol use disorder, AUD

MAOA geeni metüleeritus seoses alkoholitarvitamise häirega

Kokkuvõte

Monoamiini oksüdaas A (MAO-A) on mitokondriaalne ensüüm, mida on seostatud alkoholitarvitamise häirega (AUD). Üks MAO-A ekspressiooni mõjutavaid faktoreid on *MAOA* geeni metüleerituse tase, mida saab mõõta valgetest verelibledest. Väärtused mõõdeti 12 eraldi CpG saidile. Meessoost uuritavaid hinnati elujooksul esinenud AUD (N_{AUD} = 42, $N_{mitte\text{-}AUD}$ = 78), suitsetamise, tarbitava alkoholitüübi ja selle sageduse põhjal. Seda kolmes vanuses: 15., 18. ja 25. eluaastal. 25 aasta vanuselt olid statistiliselt olulised CpG saidid 3, 5, 7 ja 11, seda madalamate metüleerituse tasemetega AUD korral. CpG11 andis olulise tulemuse nii 18- kui ka 25-aastastel, mistõttu võiks seda pidada alkoholitarvitamisele kõige tundlikumaks. AUD-tundlike saitide puhul omas suitsetamine statistilist olulisust samuti CpG11 metüleerumise puhul. Suitsetamine interakteerus lahja alkoholi tarbimise korral CpG saidil 8 (25-aastased) kui AUD ei olnud faktor. *MAOA* genotüübi ja AUD vahel leidis aset statistiliselt oluline interaktsioon 25-aastaste CpG saitidel 3 ja 9. Üldjuhul olid MAOA metüleerituse tasemed madalamad AUD puhul kui mitte-AUD korral ning statistiliselt oluliste tulemuste arv suurenes koos vanusega.

Märksõnad. monoamiini oksüdaas A, *MAOA*, DNA metüleerimine, *MAOA* metüleerimine, alkoholitarvitamise häire, AUD

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1. Introduction

1.1 Background

Monoamine oxidase (MAO), existing in two isoforms (MAO-A and MAO-B), is a mitochondrial enzyme that catalyzes the oxidative deamination of biogenic amines, including neurotransmitters such as dopamine (DA), norepinephrine (NE), serotonin (5-HT) and histamine (Youdim & Bakhle, 2006; Yang et al., 2012; Matošić et al., 2016). In the brain, MAO-A is predominantly found in catecholaminergic neurons, and outside of it mostly in fibroblasts and placental tissue (Naoi et al. 2016). Because this enzyme is a prolific moderating agent of central the nervous system (CNS) function by regulating mood, emotion, motor, perceptual and cognitive processes, then any up- or downregulation in its levels can bring along significant effects in brain function as well as in behavioural and emotional phenotypes (Duncan et al., 2012; Naoi et al. 2016).

Alcohol use disorder (AUD)[1] is a heterogenous psychiatric condition which causal mechanisms are considered to be both environmental and genetic (Matošić et al., 2016). Because alcohol is a CNS depressant, where it mainly inhibits neuronal signaling by binding with GABA receptors and suppresses glutamate release, it interacts with variety of neurotransmitters, including DA, NE, and 5-HT (McIntosh & Chick, 2004). As MAO-A plays an important role in their metabolization, it is reasonable to presume that alcohol dependency and the activity of this particular enzyme in the brain could be linked (Tamm, 2014; Matošić et al., 2016). Low MAO-A activity, which can increase NE and 5-HT levels in brain, has among other things been liked with poor impulse control and aggression (e.g. Chester et al., 2015; Kolla et al., 2015; Naoi et al. 2016; Bartolato et al., 2018), both of which can be associated with problematic alcohol consumption (e.g. Eensoo et al., 2004; Samek et al., 2018; Park et al., 2021; Win et al., 2021).

DNA methylation is a chromatin modification, where selective addition of a methyl (CH_3) group to cytocine (C) in the CpG nucleotides (catalyzed by methyltrasferases), forms 5-methylcytosine (5-mC), which has an important role in euchromatin–heterochromatin transformations and DNA

[1] Previously differentiated as alcohol abuse disorder and alcohol dependence disorder, but since the publication of DSM-5 in 2013 considered together as one alcohol use disorder with three severity sub-classifications (NIAAA, 2021). It is important to note, that although this study generally uses AUD per classification of DSM-5, then MINI (see 2.3), which was used to identify participants for AUD, is based on DSM-IV and technically distinguishes alcohol abuse and dependence.

transcription factors binding (Zhang et al., 2021). By these means methylation is an epigenetic mechanism of DNA's transcriptional control, i.e. altering methylation levels are able to alter gene expression.

One substantial factor in the expression activity of MAO-A enzyme is the methylation of *MAOA* gene. In 2012 Shumay, Logan, Volkow and Fowler established that there is a correlation between average methylation of the *MAOA* promoter region in white blood cells and MAO-A activity in the brain ($r = –0.61$, $p < 0.001$). Several other studies after have also provided supportive evidence to this correlative finding (Pinsonneault et al., 2006; Checknita et al., 2015; Schiele et al., 2018).

The investigation of upstream variable nucleotide tandem repeat (uVNTR) (e.g. Balciuniene et al., 2002; Saito et al., 2002; Huang et al., 2007; Gokturk et al., 2008; Yang et al., 2012; Cervera-Juanes et al., 2016) has turned out to be prominent (e.g. Sabol et al., 1998; Contini et al., 2006; Pinsonneault et al., 2006; Lee et al., 2009; Schumay et al., 2012; Samochowiec et al., 2015; Naoi et al. 2016; Bendre et al., 2018). In these studies researchers have designated *MAOA*-uVNTR genotypes with ≤ 3 repeats short (S) and >3 long (L) allels (Bendre et al., 2018). Interestingly, L-allels with 3.5 or 4 repeats have shown to be transcribed 2–10 times more efficiently than 5 repeats (similarly to S-allels, i.e. 2 and 3 repeats), thus supposing an optimal length for the regulatory gene region and dividing alleles into low and high activity groups (Sabol et al., 1998). There are indicative evidence that S-allel, i.e. also low activity allel, carriers can be more vulnerable to psychiatric disorders, including AUD (Contini et al., 2006; Bendre et al., 2018).

Considering the information above, it is reasonable to deduce that AUD and *MAOA* gene expression, leading to MAO-A enzyme activity, in terms of gene methylation could be linked. Similar relationship has been recorded in women (Philibert et al., 2008). Additionally, alcohol consumption and altered gene methylation relation has been observed on other human genes (e.g. Zhou et al., 2011; Cervera-Juanes et al., 2017; Mandal et al., 2017; Ciafrè et al., 2019; Park et al., 2020).

1.2 Research design and questions

MAOA gene is sexually dimorphic (locating on X chromosome), which means it manifests differently in men and females (Checknita et al., 2021). Interestingly, men exhibit much lower average methylation while females tend to be more methylated and inter-individually dynamic through lifetime (Harro & Oreland, 2016; Ziegler & Domschke, 2018). For more conclusive investigation, this study is focused on men and all questions applied to only one sex.

Because this study searches for possible differences in *MAOA* gene methylation between men with and without AUD, I am comparing two groups who were age and sex matched (see 2.2). Non-matched variables other than AUD, which were taken into account as possible factors altering *MAOA* methylation, were *MAOA* genotype and smoking behaviour (see 2.3). Data for this analysis was provided by The Estonian Children Personality Behaviour and Health Study (ECPBHS).

To determine if *MAOA* methylation is associated with AUD, I am building my analysis on more specific questions.

1) Are MAOA gene methylation levels different in association with AUD?

MAO-A levels are near adult levels at birth (Duncan et al., 2012). *MAOA* methylation though is as well as cumulative, reversible process and because alcohol use is one factor thought to lower gene methylation and therefore its expression (Cervera-Juanes et al., 2016), it is feasible to hypothesise that methylation levels between individuals with and without AUD could be differing over time.

2) Is there any interactive connection between *MAOA* genotype (uVNTR) and methylation in the case of AUD?

MAOA-uVNTR polymorphism has been studied in relation with AUD quite thoroughly and on one hand have given results which tie low-activity allels and greater vulnerability to alcohol dependance (Saito et al., 2002; Contini et al., 2006; Bendre et al., 2018), but on the other hand show no significance (Shumay et al., 2012; Samochowiec et al., 2015). This difference may come from not specific enough research question because low-activity 3-repeat allel in relationship with other genes in case of AUD gave also different results (Huang et al., 2007). Considering *MAOA*-uVNTR

has been proposed to be associated through MAO-A with AUD (Samochowiec et al., 2015), then it is interesting to find if this polymorphism has any correlation with *MAOA* gene methylation.

3) Does smoking affect *MAOA* methylation statistically significantly?

Cigarette smoke contains MAO-A inhibitor which means this particular enzyme levels are by default lower in smokers' organism (Fowler et al., 1996). Philibert et al. found in 2009 that similar relationship also exists between smoking and *MAOA* methylation. This analysis is important to determine how much of possibly altered methylation levels to write on AUD and how much on smoking habits' accord.

4) Can smoking and type of alcohol consumed have an interaction to MAOA methylation?

Alcohol is categorized by ethanol concentration and ethanol has been shown to modify DNA histones (including their methylation) (Shukla and Lim, 2013), so putting together two factors thought to lower gene methylation could further distinguish what or if certain differences can be attributed to AUD.

1.3 Importance of the study

Although *MAOA* in relationship with alcohol (ab)use has been studied before, there are not ample results to draw definite conclusion about the ties of the gene methylation and the phenomenon. As previously stated, studies into *MAOA* methylation have been persistently contradicting, which gives this work opportunity for future researchers to weigh and compare the data in metaanalyses and/or in relationship with more developed scientific methods.

Although there are many studies into *MAOA* gene following the discovery of MAO enzymes impact on neurotransmitter metabolism in the 1950–60's (Duncan et al., 2012), comprehensive understanding has not really emerged about *MAOA* methylation and its much hypothesized relation to psychobiological phenomenons, including AUD (see 1.1). To my knowledge, this is the first study to analyze data relevant to topic over such long time period.

All this is important to better understand AUD etiology and maybe in the future use this knowledge to advance AUD treatment.

2. Method

2.1 Initial data collection

Sample. Participants of ECPBHS were chosen by computer selected from schools of Tartu city and county, Estonia (Harro, 2015). All participants (or their guardians) signed an informed consent. (ibid.). Present study did not recruit any participants but used the data recorded in ECPBHS database. ECPBHS base study was conducted by the code of ethics by Declaration of Helsinki and certified by the Ethics Review Committee on Human Research of the University of Tartu (ibid.). Participants were tested by ECPBHS at the age of 15, 18, 25 and 33 in 1998, 2001, 2008 and 2016 respectively. Only male participants were selected in accordance with research questions.

2.2 Measures

Lifetime incidence of alcohol use disorder (AUD). Participants were assessed by clinical psychologists in 2008 at the age of 25 on the basis of Mini-International Neuropsychiatric Interview (MINI. 5.0.0) (Sheehan et al., 1998) Estonian version (Shlik et al., 2002). Due to small disordered sample, past and present cases of AUD will not be differentiated.

Methylation. Methylation was measured by bisulfate method on the basis of DNA sites in the *MAOA* promoter region, i.e. "CpG" sites. Methylation was read in Würzburgis, Germany from the proportion of methylated DNA molecules as opposed to non-methylated (Ziegler et al., 2016).

Genotype. Isolated DNA (from venous blood) was processed with PCR method and analysed by electrophoresis, where 3 repeat (low-activity allel), 4 repeat (high-activity allel) and 5 repeat (also high-activity allel) genotypes became visible in separation; for more details, see Kiive et al. (2014).

Smoking habits. Smoking habits were self-reported in terms of amount and frequency. In calculations, smoking was binary value due to small sample size.

Age. *MAOA* methylation levels have thought to be age-related, but recent study by Checknita et al. (2021) did not confirm it. Whether it is or is not a factor, age is automatically controlled for by the sample and its measurement waves.

S**ex.** Sex was automatically controlled for by the parameters of research questions.

2.3 Statistical tests

Choice of tests. The main means of comparison were t-tests and Analysis of Variance (ANOVA), both one- and two-way. If data did not meet with assumptions of normality (Q-Q plots) and/or homogeneity (Levene's test), Welch's t-test was swapped out with nonparametric Mann-Whitney U test and classic ANOVA for Kruskal-Wallis test. For ANOVA, Tukey's HSD and Dunn's tests were used, depending on the nature of groups (if assumption's values were very low and/or largly fluctuating, Dunn's test was preferred). If Kruskal-Wallis gave any significant aswers, groups were further compared in pairs by Mann-Whitney U test. In case of non-parametric tests, effect sizes are considered, because they can either indicate need for analysis with higher power or small/medium effect, which won't show up with *p*-value (Fritz, Morris, & Richler, 2012).

Statistical software. All calculations were performed on free version of JASP 0.16.1 (JASP team, 2022).

3. Results

Variables, their values and group sizes, as well as mean, median and standard deviation sizes of CpGmean are found in Appendix 1.

3.1 Are MAOA gene methylation levels different in association with AUD?

I compared with Mann-Whitney U test, if MINI lifetime instance of AUD was associated with methylation levels in 15, 18 and 25 years of age.

All significant results to question whether AUD is associated with gene methylation are found in Table 1. Overall 5 different sites (CpG11 recurring in two consecutive age groups) and one CpG mean gave a significant result. Two largest differences between subjects with AUD and controls ($p < .010$) were at CpG sites 3 and 11 of 25-year-olds. The effect sizes for these sites (as well as sites 5 and 7) also surpassed $r = .24$ (McGrath and Meyer, 2006), signifying medium effect.

Table 1

Significant correlations between AUD in life and MAOA methylation levels in three age groups with Mann-Whitney U test.

Age	Variable	Median of non-AUD group	Median of AUD group	*U*	*p*	Effect size (*r*)
15	-	-	-	-	-	-
18	CpG10	.142	.107	2027.000	.032	.237
	CpG11	.840	.802	2044.500	.025	.248
25	CpG3	.075	.055	2159.500	.002	.351
	CpG5	.055	.035	1988.500	.029	.244
	CpG7	.040	.025	1966.500	.040	.230
	CpG11	.855	.755	2111.500	.004	.321
	CpGmean	.177	.154	1956.000	.046	.223

3.2 Is there any interaction between MAOA (uVNTR) genotype and AUD on methylation?

MAOA genotype (uVNTR) can vary and is further divided into high (H) and low (L) expression subtypes based on repetitions. Data was fit for one-way ANOVA, but due to overall low values of Levene's *p*, Dunn's post hoc test was preferred.

Interaction between *MAOA* genotype and AUD was detected on two times amongst 25-year-olds. First interaction occurred at CpG3 ($F(1, 115) = 6.087$, $p = .015$, having the biggest difference between non-AUD high ($M = .079$, $SD = .031$) and low genotype ($M = .076$, $SD = .044$). Second interaction took place at CpG9 ($F(1, 115) = 4.402$, $p = .038$), where high-activity allel non-AUD ($M = .107$, $SD = .049$) and AUD ($M = .085$, $SD = .058$) group were contrasted.

Table 2

Significant ANOVA interations of AUD and MAOA genotype on methylation as well as Dunn's post hoc test specifications of statistically differing sub-groups.

Age	**Variable**	***F***	***p***	**Post hoc comparison**	***Z***	***p***
15	-	-	-	-	-	-
18	-	-	-	-	-	-
25	CpG3	6.087	.015			
				L_0 – H_0	3.866	< .001
				L_0 – H_1	2.856	.002
				H_1 – L_1	-2.268	.012
	CpG9	4.402	.038			
				H_0 – H_1	1.977	.024
				H_1 – L_1	-1.678	.047

Note. Post hoc column values are: H – high activity uVNTR, L – low activity uVNTR, 0 – no AUD, 1 – with AUD.

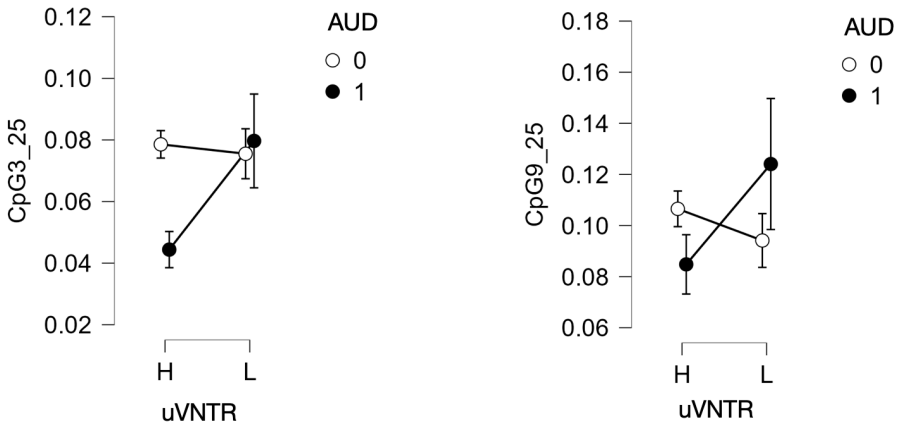

Figure 1. CpG sites 3 and 9 of 25-year-olds, where AUD and uVNTR interaction was significant. On x-axis, high- and low-activity allels are shown, on y-axis CpG site specific methylation levels and AUD is present in black dots (white is non-AUD group.

3.3 Does smoking affect methylation?

The Mann-Whitney U test was conducted to determine whether smokers' *MAOA* gene methylation is statistically different from their non-smoking peers'. Analysis was separately carried out in three age groups of 15, 18 and 25.

The statistical difference came from CpG11 of 25 years old smokers (*Mdn* = .865) and non-smokers (*Mdn* = .770), $U = 1622.500$, $p = .008$.

Because of the nature of non-parametric calculations, effect sizes (r) can have their benefits. McGrath and Meyer (2006) have proposed that even as low as $r = .10$ can in some fields of research represent small and $r = .24$ medium effect size. Based on this (see Table 3), CpG sites 6, 8, 10 and 11 could indicate small effect of tobacco smoking on 15-year-olds, CpG sites 2 and 10 small effect on 18-year-olds, and CpG sites 1, 4, 5, 6, 7, 8 as well as overall mean small effect, while CpG11 even a medium effect on 25-year-olds.

Table 3

Significant effect sizes with Mann-Whitney U test for current tobacco smoking to MAOA gene methylation in three age groups.

	Effect size (*r*)		
Variable	**15-year-olds**	**18-year-olds**	**25-year-olds**
CpG1			0.132
CpG2		0.138	
CpG4			0.192
CpG5			0.185
CpG6	0.139		0.174
CpG7			0.136
CpG8	0.223		0.174
CpG10	0.170	–0.123	
CpG11	0.103		0.311
CpGmean			0.129

3.3.1 Is methylation in AUD-sensitive CpG sites also associated with tobacco smoking?

A two-way ANOVA was conducted to examine the effect of smoking and type of alcohol (light alcohol vs. hard liquor) consumed to *MAOA* methylation levels, more specifically to previously established AUD-sensitive CpG sites 3, 5, 7, 10, 11. Frequencies were not accounted for. Analysis was performed only on 18-year-olds, because the data of 15-year-olds was tainted and almost all 25-years-old, who consumed alcohol, consumed both kinds. Tukey's post hoc test was applied.

No significant results were obtained.

3.3.2 Is there an effect of smoking and type of alcohol consumed to any CpG sites if AUD is not accounted for?

A two-way ANOVA was conducted to investigate if type of alcohol consumed and smoking in the last 30 days can statistically significantly be associated with gene methylation levels. Analysis was performed on the self-reported data of 18 years old[2] men and their methylation levels.

[2] This question was present only that year.

Significant result proved to be alcohol and tobacco interection at CpG12, $F(2, 71) = 3.778$, $p = .028$, but Tukey's post hoc did not provide any further significant contrasts.

3.4 Is there an effect of light alcohol, it's frequency of consumption and smoking to *MAOA* methylation levels?

A two-way ANOVA was conducted to investigate if light alcohol, it's frequency of consumption and/or smoking can statistically significantly affect gene methylation levels. Analysis was implemented in groups of 18- and 25-year-olds.

Interaction of light alcohol and smoking came up at 25-year-olds CpG8, $F(2, 94) = 3.661$, $p = .029$, $\eta^2 = .070$. Tukey's post hoc comparison disclosed the biggest difference to be between smokers who consumed light alcohol on semi-regular basis ($M = .134$, $SD = .092$) and smokers who consumed light alcohol on regular basis ($M = .083$, $SD = .040$).

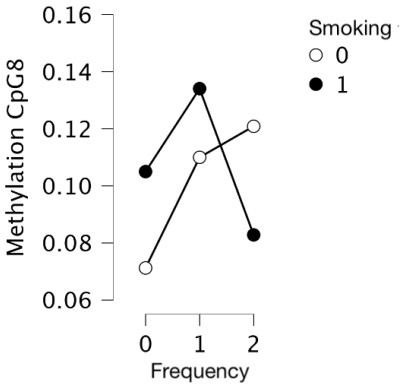

Figure 2. CpG8 methylation levels (y-axis) in association with smoking (0-no, 1-yes) and light alcohol consumption frequency (0-none, 1-semi-regular, 2-regular) on x-axis.

3.4.1 Is there any effect of hard liquor, it's frequency of consumption and smoking to *MAOA* methylation levels?

A two-way ANOVA was conducted to investigate if hard liquor, it's frequency of consumption and/or smoking can statistically significantly affect gene methylation levels. Analysis was made on 25-year-olds, as data for 15 years old was tainted and many 18-year-olds had chosen to not answer that question, therefore diminishing answerers' group sizes too small to account for.

There were no statistical differences or interactions between hard liquor consumption and/or smoking on methylation levels.

4. Discussion

4.1 Interpretation

Although any of these results did not reveal any noteworthy CpG sites with persistent changes in values in association to AUD, few CpG sites were recurring nonetheless (see Table 4). CpG sites 3, 10, 11 and 12 gave all two or more hits over the inquiries in this paper, where both CpG11 and CpG12 even crossed age gaps. On two occasions, overall amplicon methylation (CpGmean) gave an hit in some kind of association to alcohol consumption, AUD not accounted for. CpG values tended to be lower, when alcohol had a role in one's lifestyle.

When analysing *MAOA* methylation levels in three different ages in relation to AUD, then the older the sample, more associations were found. When 15 years old group had no indication of future AUD in their methylation levels, 18-year-olds had already two significant sites: CpG10 and 11. At 25, three new sites, as well as overall mean, with significant results were added (CpG3, 5, 7), CpG11 persisted and CpG10 lost its interaction. Growing correspondence between statistically lower CpG site methylation and AUD occurrence could show methylation's cumulative nature, CpG11 being the most sensitive to disordered alcohol use.

MAOA genotype and AUD had an interaction at CpG sites 3 and 9 of 25-year-olds. CpG3 methylation was almost the same for AUD and non-AUD group, but H-allel had the biggest difference. CpG9 values were almost inverted between groups, where non-AUD had lower methylation with L-allel and AUD group significantly higher methylation with the same genotype. In both cases AUD seemed to raise L-allel methylation drastically.

Interestingly, smoking alone did not seem to greatly influence *MAOA* methylation levels, only result coming from CpG11 of 25-year-olds. Because performed tests were non-parametric, I considered effect sizes additionally and those in itself can indicate small effect of smoking to gene surface. Even based on effect sizes, no CpG site seemed consistent over the age groups in association to tobacco smoking, although CpG10 I find peculiar, because of it's almost inverted effect size from ages 15 to 18. Similar to uVNTR findings, effect sizes of smoking were most numerous at 25, again

pointing to methylation's cumulative nature (smokers at 25 years were also largely smoking prior to this).

Although smoking alone did not seem too significant, AUD-sensitive sites (CpG3, 5, 7, 10, 11) were further analysed for possible interactions between smoking and type of alcohol consumed. No significant values came up, but when expanding to CpG sites previously left out, CpG12 was found significant; no sub-groups differed significantly according to post hoc.

To be sure, that smoking or alcohol type aren't too big of a factors in this type of study, methylation was then analysed for light alcohol and smoking as well as hard liquor and smoking interaction. From questionnaire, it was apparent, that consumption of light alcohol is prevalently habitual amidst 18–25 years old Estonian men. Significant interaction between smoking and light alcohol proved to be at CpG8 of 25-year-olds, more specifically between smokers of semi-regular and regular light alcohol consumers. When smoking together semi-regular light alcohol had the highest methylation, then smoking along regular light alcohol had the second lowest CpG8 methylation levels (the lowest being non-smoking-non-drinking group). Similar inquiry into the relationship of smoking and hard liquor yielded no such, or any, results. Because I based my initial question on the amount of ethanol consumed, it could be unessential hypothesis or the summative ethanol of light alcohol exceeds the total amount of ethanol consumed in the form of hard-liquor.

Table 4

CpG sites and how many times they gave a statistically significant result in separate age groups over the course of this research.

	Times given a significant result		
Variable	15-year-olds	18-year-olds	25-year-olds
CpG1			1
CpG3			2
CpG5			1
CpG7			1
CpG8			1
CpG9			1
CpG10		2	
CpG11		2	3

CpG12	1	2	
CpGmean		1	1

4.2 Conclusion

AUD indeed seems to lower *MAOA* methylation, but that on CpG sites individually, depending on age. Because methylation is cumulative (Cervera-Juanes et al., 2016), which this study can support (number of significant results increased with age), it should also be reversible (some significant sites while younger lost their significance in older age). Results suggest that *MAOA* genotype can change methylation levels in AUD group, which can be tied to previous hypothesis, that low-activity allel individuals could be more vulnerable to AUD than high-activity genotype (Saito et al., 2002; Contini et al., 2006; Bendre et al., 2018). Smoking in this research showed to be insignificant, but due to more concrete evidence, that smoking does affect methylation (Fowler et al., 1996; Philibert et al., 2009), I would challenge my own work. Smoking was relevant in interaction with light alcohol, but AUD was not accounted for.

4.3 Limitations

For examining associations between AUD and methylation, the sample is very small ($N_{\text{no-AUD}} = 78$, $N_{\text{AUD}} = 72$), especially when making further differentations like smoking. In case of smoking, non-smoking subjects with AUD can be underrepresented ($N_{\text{age-25}} = 11$). The data also suffers from the nature of self-report questionnaire, because participants regularly choose to skip some answers, therefore eliminating themselves from further analysis.

To avoid groups with very few subjects, factors were reduced to binary values (yes/no). But in reality, this puts once a month cigarette smokers into the same group with everyday 10+ cigarette smokers, thus broadening possibly significant values into spectrum. With alcohol consumption, I could only take into account the type or frequency, because adding both as variables reduced group sizes as low as one individual or none.

As memory writes itself over (viide), self-reported values for freguencies and amount can be inaccurate. For example, answers to the same question (e.g. "How old were you when you first tried at least half a dose of alcohol?") changed every year.

4.4 Recommendations

Because AUD is more manageable disease than, e.g. many aggressively occurring neuro- or skeletal diseases of genetic defects, study in this vein could be broadened to bigger non-AUD sample and used as preventative education against alcohol overuse. Examples of behavioural, motivational and/ or emotional outcomes in association with altered methylation levels with the help of other science fields' scientists would make even greater impact.

When conducting research in the case of AUD, much bigger samples would be beneficial to even this simple of an analysis. When building research design, less questions would be better, because as seen from ECPBHS sample, people start skipping questions when there are many. Age matching would maybe be better done in intervals, so more subjects are found. Data won't necessarily be longtidudal, because methylation can be reversible as well as cumulative and this can be seen from answers to questionnaire. With concise questionnaire, no follow-up appointment needed and available equipment, data collecting could even be done in clinics.

On the same data used here, different and/or more complex analyses should be applied. When hypothesis is established in some direction ("methylation levels are lower in AUD and/or smoking population"), linear regression would be useful. Abundant data from ECPBHS would even make possible some kind of case study, which could unveil some more specific factors.

Appendices

Appendix 1

Measure types, group sizes and overall mean CpG methylation indicators amongst these groups.

				CpGmean		
Measure	Possible values	Age	Group size	Mean	Mdn	SD
Methylation	0 ... 1	15	$N = 116$	.164	.165	.058
		18	$N = 120$	.177	.176	.037
		25	$N = 119$	.172	.173	.047
MAOA genotype	L (low), H (high)	15	$N_L = 44$	.154	.152	.063
			$N_H = 72$	.171	.166	.054
		18	$N_L = 44$	.175	.174	.046
			$N_H = 76$	.178	.181	.032
		25	$N_L = 43$	.172	.161	.036
			$N_H = 76$	.173	.174	.050
Lifetime incidence of AUD	0 (no), 1 (yes)	15	$N_0 = 76$	.167	.164	.059
			$N_1 = 40$	.160	.166	.055
		18	$N_0 = 78$	.182	.177	.037
			$N_1 = 42$	.167	.173	.037
		25	$N_0 = 78$	.176	.177	.039
			$N_1 = 41$	.166	.154	.060
Smoking habits	0 (no), 1 (yes)	15	$N_0 = 97$	.164	.165	.062
			$N_1 = 19$	.165	.166	.027
		18	$N_0 = 56$	.179	.178	.032
			$N_1 = 37$	.183	.183	.046
		25	$N_0 = 45$	.177	.175	.052
			$N_1 = 55$	.169	.163	.049
Alcohol type	0 (none), 1 (light alcohol), 2 (hard liquor)	15	-	-	-	-
		18	$N_{\text{no-alcohol}} = 12$	.192	.185	.038
			$N_{\text{light alcohol}} = 28$	.175	.176	.031

			$N_{\text{hard liquor}}$= 37	.178	.178	.035
		25	-	-	-	-
Frequency	0 (none), 1 (semi-regular), 2 (regular)	In case of light alcohol:				
		15	-	-	-	-
		18	$N_0 = 12$	.189	.185	.043
			$N_1 = 23$	.180	.183	.028
			$N_2 = 40$	.174	.174	.035
		25	$N_0 = 6$	.162	.154	.033
			$N_1 = 34$	.184	.178	.053
			$N_2 = 60$	.167	.163	.049
		In case of hard liquor:				
		15	-	-	-	-
		18	-	-	-	-
		25	$N_0 = 8$	.164	.161	.029
			$N_1 = 74$	.173	.174	.045
			$N_2 = 18$	.175	.171	.075

Note. Light alcohol is here considered less than 20‰ and hard liquor over this value. Semi-regular means that consumption takes place less than once per week and regular meaning at least once per week.

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