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Short-term changes in global methylation and hydroxymethylation during alcohol detoxification

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Abstract
Alcohol dependence is a common public health problem and epigenetics may offer new aspects in understanding the biological and genetic underpinnings and improve treatment of this complex disease. Supposedly, methylation and hydroxymethylation are altered in brain tissues and in synapse-related genes due to chronic alcohol intake and during withdrawal. To assess potential epigenetic changes after cessation of chronic alcohol intake, we compared 23 alcohol-dependent individuals during inpatient alcohol detoxification with 13 carefully matched controls. Blood samples were taken on the day of admission, after one and after two weeks at the end of inpatient treatment. Genome-wide global methylation and global DNA hydroxymethylation were compared across groups. There were significant differences in global methylation across time from admission to one and two weeks of inpatient withdrawal ($p < 0.001$). These findings were paralleled to changes in global DNA hydroxymethylation across time when age was employed as a cofactor ($p < 0.001$). Several potentially influencing variables like severity of withdrawal, dose of withdrawal medication and alcohol intake before admission did not

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yield significant influence on epigenetic changes. The results confirm previous findings of significant alterations of epigenetic patterns during alcohol intoxication and present for the first time hydroxymethylation changes in these individuals.

1. Introduction

In Germany, some 1.8 million individuals are dependent on alcohol, and additional 1.6 million persons use alcohol in a harmful way (Batra et al., 2016). One of the major reasons why alcohol-dependent individuals enter treatment is alcohol withdrawal. The etiology of alcohol dependence and related phenotypes like alcohol withdrawal is certainly complex and include an interaction of various environmental and biological or genetic factors. This interaction may be at least in part reflected by epigenetic changes during alcohol withdrawal.

The acute treatment of alcohol withdrawal usually starts with detoxification (Mann et al., 2017). Many previous reports note that alcohol intoxication and subsequent withdrawal is related to several biological and epigenetic changes.

There is growing literature on genetics and epigenetics of alcohol withdrawal (Nieratschker et al., 2013). Accordingly, epigenetic characteristics are not associated with specific cell type composition and require no correction for cellular heterogeneity (Farre et al., 2015). While in humans, epigenetic patterns of post-mortem brain tissue permit the analysis of epigenetic patterns only once, peripheral tissue, particularly blood, provides the advantage of being able to sample DNA and probe methylation at various time points with respect to alcohol use, including those after recent use and after periods of withdrawal (Tulisiak et al., 2017).

Alcohol use disorders are also known to be associated with DNA methylation changes (Zhang and Gelernter, 2017). In the animal model, effects of acute alcohol on amygdala signaling pathways via epigenetic remodeling were reported (Pandey et al., 2017). The effects of alcohol detoxification on methylation status of individual CpG sites and serum levels of vasopressin and atrial natriuretic peptide have been demonstrated (Glahn et al., 2014), as well as changes in epigenetic regulation of GATA4-dependent brain natriuretic peptide expression (Glahn et al., 2016). Chronic alcohol consumption was reported to be associated with altered methylation patterns of various genes acting via s-adenosylmethionine (SAM) metabolism and altering homocysteine levels (Krishnan et al., 2014). Another relevant epigenetic mechanism due to high frequency alcohol intake and withdrawal is hydroxymethylation of gene CpG islands (Kato and Iwamoto 2014). Hydroxymethylation was discovered as a new constituent of mammalian DNA. Hydroxymethylation replaces, at the C5-position in cytosine, the hydrogen atom with a hydroxymethyl group (Munzel et al., 2011). In contrast to CpG methylation which results in lower gene expression, hydroxymethylation in intragenic regions is associated with higher gene expression (Kato and Iwamoto, 2014).

Overall alcohol and epigenetics seems to be a complex system which involves alcohol-induced epigenetic changes and potential counter-mechanisms. There are many hitherto unknown effects of alcohol detoxification in the field of epigenetic in peripheral blood. The aims of this study are first to confirm changes in global methylation and second, to investigate global hydroxymethylation during alcohol detoxification in male alcohol-dependent individuals. As a comparison group, healthy age-matched male controls were enrolled. Since previous research demonstrated that chronic alcohol intake increases global methylation patterns, we hypothesize that alcohol intake before withdrawal influences global methylation (review by Zhang and Gelernter 2017). Correlations between long-term alcohol consumption and methylation of six selected genes was reported (Weng et al., 2015). Previous research also indicated a significant alteration of vasopressin (AVP) and atrial natriuretic peptide (ANP) CpG sites during withdrawal (day 1 to day 14) (Glahn et al., 2014).

Potential influencing factors for hydroxymethylation patterns during withdrawal are hitherto unknown. We hypothesize that these patterns change over time and are influenced by severity of withdrawal and dose of withdrawal medication, since this mechanism are considered to counter methylation changes.

2. Experimental procedures

2.1. Alcohol-dependent individuals

Treatment-seeking inpatient alcohol-dependent subjects were recruited from an addiction treatment ward of the Ludwig-Maximilians-University of Munich, Germany.

Subjects were excluded if they had any current and acute Axis I psychiatric disorder, other than alcohol and nicotine dependence. The psychiatric hospital offers an inpatient detoxification treatment program, which includes somatic detoxification, individual and group psychotherapy, counselling for social and financial problems as well as somatic medical care for 15 days. This approach is in line with the German S3-guidelines for inpatient treatment (Mann et al., 2017). All recruited individuals received stepwise pharmacological treatment with Oxazepam depending on the severity of their alcohol withdrawal.

All patients were unrelated, of German descent, older than 18 years and met ICD-10 and DSM-IV criteria for alcohol dependence, assessed with a structured clinical interview SSAGA (Semi-Structured Assessment on the genetics in alcoholism) (Bucholz et al., 1994; Wittchen et al., 1997). The diagnostic assessment was performed without knowledge of genotype data. Demographic variables and clinical characteristics are presented in Table 1.

Blood samples were taken on the day of admission in the hospital, after seven and after 15 days of inpatient treatment.

Informed written consent was obtained from all patients and controls after complete and extensive description of the study, which was approved by the Ethics Committee of the Ludwig-Maximilians-University of Munich in accordance with the principles laid down in the Helsinki Declaration (1964).

Table 1 Demographic variables and clinical characteristics of alcohol- dependent patients and healthy controls.

	AD patients	Controls	*p*
Gender (males / females)	23 / 0	13 / 0	
Age (years, mean ± SEM)	49.04 ± 10.5	41.31 ± 12.9	0.06
Age of onset (years, mean ± SEM)	32.66 ± 9.76		
Mean daily alcohol intake before admission (g/d)	113.33 ± 46.8		
Duration of dependence (years, mean ± stddev)	16.23 ± 11.5		
Number of current withdrawal symptoms	2.95 ± 2.27		
Number of positive DSM IV criteria (mean ± SEM)	5.57 ± 1.55		
AD patients with DT liftetime history, n / total (%)	4 (17%)		
AD patients with WS liftetime history, n / total (%)	7 (30%)		
Current smokers	12/11 (52%)		

Abbreviations are: AD, alcohol dependence; DT, delirium tremens; WS, withdrawal seizures, SEM, standard error of mean.

2.2. Control group

The control group was recruited from the general population at different locations (e.g. libraries, road construction sites and department stores) intending to represent a range of social classes from university graduates to unskilled workers. All subjects underwent comprehensive medical and psychiatric assessment to exclude persons with possible psychiatric Axis I/II disorders, such as schizophrenia, depression, personality disorders and alcohol and substance use disorders. Subjects were excluded if they had any current Axis I disorder, substance other than nicotine use disorders, or any physical illness. None of the subjects were related, all were of German descent and did not report any first-degree relatives with any mental disorders.

Blood samples were taken on day one, after seven days and after 15 days similar to the inpatients during withdrawal. Informed written consensus was obtained as well.

2.3. Analysis of DNA methylation

Genomic DNA was extracted from whole blood according to standard methods.

Global 5-methylcytosin (5-mC), as well as 5-hydroxymethylcytosin (5-hmC) methylation was measured as instructed by the manufacturer, using ELISA kits for 5-mC (Nr. D5325, Zymo Research, Irvine, USA) and 5-hmC (Nr. D5425 Zymo Research, Irvine, USA).

All samples were assayed in duplicates and the same plate was used. Detection was done on a Polarstar Optima Plate Reader (BMG Labtech, Ortenberg, Germany).

2.4. Statistical analysis

Statistical analyses of the clinical and epigenetic data results were conducted using IBM SPSS Statistics Version 21 for Windows (SPSS Inc., Chicago, IL, USA).

The methylation level of each subject was defined as the average percentage of methylated and hydroxy-methylated cytosins from all assessed CpG sites. All parameters did not yield significant deviation from normal distribution as calculated by Kolmogorov-Smirnov non-parametrical tests.

As the mean methylation and hydroxy-methylation levels yielded no deviation from normal distribution, general linear models (GLM) were used to compare the global mean methylation across time-points with alcohol-dependent patients vs. controls as the between-subjects factor and age as a covariate.

For assessment of methylation changes during withdrawal, a GLM repeated-measures analysis was performed for patients only.

Further, the influence of several potential confounders was considered in the analysis. For the global methylation over time, the potential influence of withdrawal medication (dose of total oxazepam mg during withdrawal) and severity (SSAGA sum of acute withdrawal symptom items) were considered. Global hydroxymethylation patterns were analyzed for the influence of alcohol intake before admission (g/d) and duration of alcohol dependence (years). GLM covariance statistics were employed to assess influence of hypothesized confounders. Correction for multiple testing was employed if needed.

Data are presented as mean +/− standard-deviations in Figs. 1 and 2.

3. Results

A total of 23 alcohol-dependent individuals during detoxification as well as 13 healthy controls were included. Characteristics of alcohol-dependent individuals and healthy controls are presented in Table 1. All alcohol-dependent individuals and healthy controls were male. A significant difference in age was detected as healthy controls had a mean age of 41.31 while alcohol-dependent individuals had a mean age of 49.04 years.

However, age did not influence methylation (F-value 0.107, df 1; $p = 0.746$), while hydroxymethylation (F-value 5.937, df 1; $p = 0.020$) patterns were age-dependent.

Therefore, age was employed as a co-factor in subsequent analyses of hydroxymethylation patterns.

In Fig. 1 global methylation status of alcohol-dependent individuals at time of admission, one week and two weeks after admission in comparison to healthy controls are illustrated. For the three time points, we evaluated global methylation status and analyzed potential differences. Significant alterations in methylation status were detected across time points. Difference between alcohol-dependent individuals and healthy controls increased with time (F-value 20.157, d 1, $p < 0.001$).

Fig. 2 shows the global DNA hydroxymethylation across groups and time. Using age as a co-variate, there is a statistical significant difference of hydroxymethylation across time (F-value 7.566, d 1, $p = 0.009$).

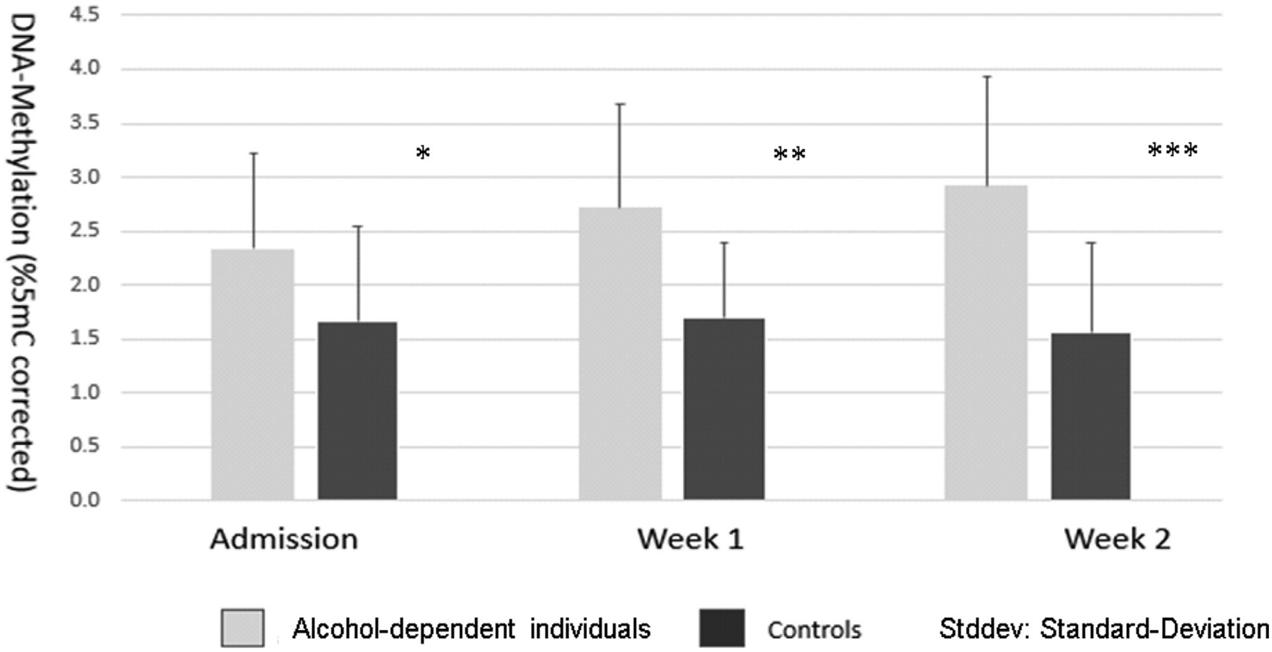

Fig. 1 Global methylation in alcohol-dependent individuals vs. controls across time-points
*: $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

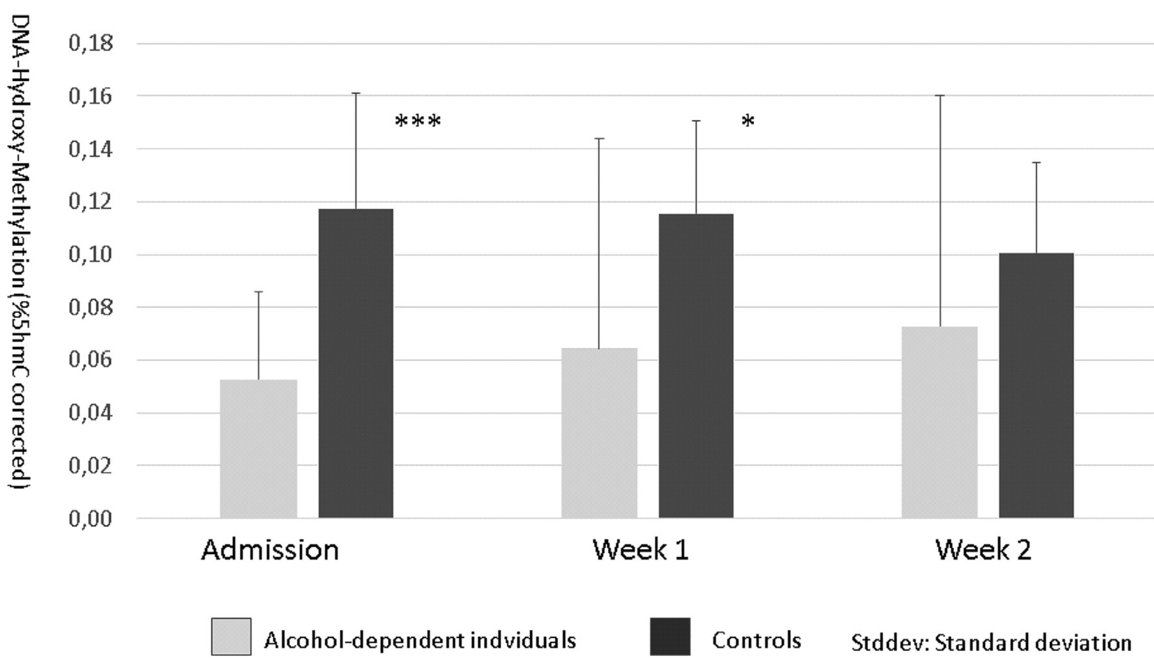

Fig. 2 Global hydoxy-methylation in alcohol-dependent individuals vs. controls across time-points
*: $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.1. Influence of potential confounders

No significant influence of withdrawal severity (F-value 1.69; df 1, $p = 0.208$) or oxazepam dose during withdrawal (F-value 2.49; df 1, $p = 0.130$) was detected by analysis of covariance on methylation patterns over time.

A statistical trend (F-value 4.190, df 1, $p = 0.063$) was detected for alcohol intake before admission while duration of alcohol dependence did not yield any statistical significance (F-value 0.586, df 1, $p = 454$).

Alterations of global hydroxymethylation were not influenced by alcohol intake before admission (F-value 0.139, df

1, $p = 0.716$) and duration of alcohol dependence (F-value 0.453, df 1, $p = 0.510$) when age was used as a co-factor.

Withdrawal severity (F-value 0.633, df 1, $p = 0.436$) and Oxazepam dose (F-value 0.055, df 1, $p = 0.817$), did not influence hydroxymethylation over time.

Among the patients, $n = 12$ are current tobacco users (52%). They consumed an average of 24.4 ± 14.4 (range 5-20) cigarettes per day. Smoking status and number of cigarettes consumed did not have any influence on hydroxymethylation patterns (F-value 0,673, $p = 0.579$).

4. Discussion

Results of this study on epigenetic mechanisms during alcohol withdrawal are in line with previous findings which demonstrated significant changes of global methylation patterns over a period of two weeks. Several studies of individuals during alcohol withdrawal reported methylation changes, mainly in specific CpG sites of candidate genes. Bönsch et al. (2004) analyzed the relationship between global DNA methylation, plasma homocysteine, and alcohol dependence (AD) in a case-control sample (90 AD cases and 89 healthy controls). A significant increase (by 10%) of global DNA methylation in AD subjects who had elevated levels of homocysteine was detected. Kim et al. (2016) evaluated methylation levels of the repetitive element Alu in peripheral blood DNAs of 135 AD patients vs. 150 healthy controls. Again, significantly higher methylation levels of Alu in AUD patients compared to healthy controls were reported. Regarding specific candidate genes, Bönsch et al. (2005) found hypermethylation at the alpha-synuclein gene (SNCA) promoter. Two other studies demonstrated an elevated methylation at the dopamine transporter (DAT) (Hillemacher et al., 2009b) and vasopressin (AVP) genes (Hillemacher et al., 2009a). There was also increased methylation measured at the OPRM1 gene, which encodes for the μ-opioid receptor (Zhang et al., 2012), a gene significantly involved in alcohol reward pathway. The brain-derived neurotrophic factor (BDNF) gene codes for a protein involved in cell health and promotion of synaptic growth and differentiation, and methylation at its promoter was increased in blood of alcohol-dependent patients but returned to baseline after 14-day withdrawal (Heberlein et al., 2015). Blood from alcohol-dependent males demonstrated an increase in methylation of the nerve growth factor (NGF) promoter between withdrawal days 7 and 14 and corresponding reduced transcription of that gene (Heberlein et al., 2013), although there was no change in NGF methylation from withdrawal days 1–7 in the same subjects.

However, the reports on increased methylation in AD individuals are not consistent. Two other studies, one with 161 healthy control subjects (Zhang et al., 2011) and another with 384 healthy women (Ono et al., 2012) failed to identify a significant effect of alcohol drinking on global methylation of blood DNAs. Manzardo et al. (2012) examined global DNA methylation patterns in postmortem frontal cortex tissues of 10 AUD patients and 10 matched controls using methylation DNA immunoprecipitation and microarray assays, but no significant global DNA methylation differences between AUD patients and control subjects were observed.

Two Epigenome-wide association studies (EWAS) on AUDs were conducted (Philibert et al., 2014, 2012). Initially, they examined the impact of recent alcohol use on DNA methylomes using lymphoblast DNAs derived from 165 female adoptees and found widespread changes in DNA methylation across the genome due to recent alcohol consumption (Philibert et al., 2012). In a subsequent EWAS with 33 AD patients and 33 controls, widespread DNA methylation changes in the peripheral blood of AD subjects were detected, and these changes were mapped to gene networks involved in apoptosis (Philibert et al., 2014).

Methylation changes have also been observed in individual genes related to neurogenesis, inflammation, and alcoholism, with an overall trend of hypermethylation (Weng et al., 2015). In the current study, global hypermethylation increases during withdrawal. These changes were influenced by alcohol consumption prior to admission at a statistical trend level and may reflect long-term effects of chronic alcohol intake on global methylation patterns rather than severity of alcohol withdrawal itself or dose of benzodiazepines (oxazepam) administered during withdrawal.

Further, only two studies are hitherto known to have used the longitudinal DNA methylation measures to identify DNA methylation changes associated with chronic alcohol consumption. One study analyzed AVP and ANP promoter DNA methylation changes in the peripheral blood of 99 AUD patients short-term on days 1, 7, and 14 of alcohol withdrawal (Glahn et al., 2014) and another study tracked blood DNA methylomic changes in 10 AUD patients (normal in phases 1 and 2 but affected with AUDs in phase 3) and 10 controls (unaffected with AUDs) in three phases (phase 1: 1986; phase 2: 1990–1992; and phase 3: 2003–2009) (Weng et al., 2015). These two longitudinal studies support that alcohol consumption influences DNA methylation, and genome-wide DNA methylation drifts over time.

Differences in global methylation across studies may also depend on the time point of assessing epigenetic patterns in patients. As we observed an increase of methylation during withdrawal, global methylation changes may not be attributable to current amount of alcohol intake, severity of withdrawal symptoms or other environmental factors or a combination of these.

In comparison, hydroxymethylation was significantly lower in alcohol-dependent individuals at the start of withdrawal compared to controls. In the two subsequent time points, while still at a significantly lower percentage compared to controls, a rise of hydroxymethylation was observed. Contrary to our hypothesis, no influence of any potential confounders, except age, was found, i.e. alcohol intake before admission, length of alcohol dependence, severity of withdrawal or oxazepam dose taken.

Thus, there is a parallel increase of both methylation and hydroxymethylation during alcohol withdrawal. It is not clear whether hydroxymethylation serves as a potential compensatory downstream mechanism to cope with increased methylation and subsequent lower gene expression, or depends on factors independent of methylation during alcohol withdrawal.

In contrast to DNA methylation studies, the functional and pathophysiological roles of hydroxymethylation have only recently been proposed. Hitherto, there are few studies in human brain on hydroxymethylation. Most of studies on hydroxymethylation focus on ES cells (review by Kato and Iwamoto, 2014). ES cells were found to contain high levels of hydroxymethylation which decreases after differentiation (Kinney et al., 2011; Szwagierczak et al., 2010).

To our knowledge, hitherto no human study on the relation between hydroxymethylation in individuals with alcohol use and alcohol use disorders (vs. controls) were conducted. There is some evidence from animal research. A recent study reported the role of DNA methylation regulating medial prefrontal cortex (mPFC) gene expression and alcohol-related behaviors in rats, which were 3 weeks in abstinence following alcohol dependence (Barbier et al., 2015). Post-dependent rats showed increased alcohol intake, which was associated with increased DNA methylation as well as decreased expression of genes encoding synaptic proteins involved in neurotransmitter release in mPFC. Interestingly, probing of the whole transcriptome from this experiment also revealed statistically significantly decreased expression of Tet1 and Tet3 transcripts, suggesting alcohol-related changes in DNA hydroxymethylation. This observation may support the results of our study.

Moreover, Tammen et al. (2017) investigated genomic and gene-specific hydroxymethylcytosine patterns in alcohol consuming mice. They found alterations in the hydroxymethylcytosine patterns, which was partially supported by altered mRNA expression. These results suggest that changes in hydroxymethylation may play a role in the liver's response of mice to alcohol.

Several limitations of the study have to be mentioned. First, the sample is relatively small which limits generalization and prompts replication studies. Second, not all potential confounding factors could be accounted for, i.e. alcohol blood level on admission, potential alcohol relapses during withdrawal and use of other substances or tobacco. However, recent studies reported that global blood DNA methylation and comorbid alcohol and nicotine use was positively correlated and result both in hypermethylation (Semmler et al., 2015). In our sample, no influence of smoking status on hydroxymethylation was found.

Further, since this sample is a pilot study, we enrolled males only to avoid potential gender effects on hydroxymethylation patterns. Subsequent recruitment also plans to include female patients. Furthermore, in previous research, alcohol-related methylation differences were observed in males only. Wang and colleagues used Illumina HumanMethylation450 BeadChip assays to perform whole genome methylation profiling in the prefrontal cortex of alcoholics and control cases. They found 1812 differentially methylated CpGs (including non-promoter CpGs found in gene bodies and 3′ UTRs) after multiple comparison correction, 66.3% of which were hypermethylated in alcoholic subjects (Wang et al., 2016). Remarkably, differences in DNA methylation were only observed in male subjects, hinting at gender differences in alcohol-induced DNA methylation in brain.

Epigenetic patterns were analyzed from peripheral blood samples and not from liquor or brain tissue. However, peripheral tissue, particularly blood, provides the advantage to sample DNA and probe methylation at various time points, for instance during alcohol withdrawal (Andersen et al., 2015).

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Contributors

UWP designed the study and performed the statistical analyses. GK wrote the first draft of the study and supervised the writing. KA, CW, AK and MNT recruited participants and performed the measurements. PZ performed the laboratory analyses and MS, DK, WB, FK and LP assisted with literature search, interpretation of data and elaborated the written report. All authors contributed to and have approved the final manuscript.

Conflict of interest

All authors declare no conflict of interest.

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