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Acute alcohol withdrawal and recovery in men lead to profound changes in DNA methylation profiles: a longitudinal clinical study

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ABSTRACT

Background and Aims Withdrawal is a serious and sometimes life-threatening event in alcohol-dependent individuals. It has been suggested that epigenetic processes may play a role in this context. This study aimed to identify genes and pathways involved in such processes which hint to relevant mechanisms underlying withdrawal. **Design** Cross-sectional case–control study and longitudinal within-cases study during alcohol withdrawal and after 2 weeks of recovery **Setting** Addiction medicine departments in two university hospitals in southern Germany. **Participants/Cases** Ninety-nine alcohol-dependent male patients receiving in-patient treatment and suffering from severe withdrawal symptoms during detoxification and 95 age-matched male controls. **Measurements** Epigenome-wide methylation patterns were analyzed in patients during acute alcohol withdrawal and after 2 weeks of recovery, as well as in age-matched controls using Illumina EPIC bead chips. Methylation levels of patients and controls were tested for association with withdrawal status. Tests were adjusted for technical and batch effects, age, smoking and cell type distribution. Single-site analysis, as well as an analysis of differentially methylated regions and gene ontology analysis, were performed. **Findings** We found pronounced epigenome-wide significant [false discovery rate (FDR) < 0.05] differences between patients during withdrawal and after 2 weeks [2876 cytosine–phosphate–guanine (CpG) sites], as well as between patients and controls (9845 and 6094 CpG sites comparing patients at time-point 1 and patients at time-point 2 versus controls, respectively). Analysis of differentially methylated regions and involved pathways revealed an over-representation of gene ontology terms related to the immune system response. Differences between patients and controls diminished after recovery ($>$ 800 CpG sites less), suggesting a partial reversibility of alcohol- and withdrawal-related methylation. **Conclusions** Acute alcohol withdrawal in severely dependent male patients appears to be associated with extensive changes in epigenome-wide methylation patterns. In particular, genes involved in immune system response seem to be affected by this condition.

Keywords Alcohol, DNA methylation, epigenetics, longitudinal study, substance use disorder, withdrawal.

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INTRODUCTION

Alcohol use disorder (AUD) is a complex, heterogeneous disease that poses significant burdens to public health [1]. It is among the most prevalent mental disorders worldwide, and contributes substantially to global morbidity and mortality [2]. AUD is highly disabling, and associated with many physical and psychiatric comorbidities. It is

characterized by compulsive and uncontrolled drinking and frequent relapses during abstinence phases. AUD patients usually develop pronounced tolerance that is characterized by an increased amount of alcohol required to achieve the same effect, i.e. the attempt of the body to restore its normal function under ethanol exposure. If drinking is discontinued mental and physical symptoms appear, named the alcohol withdrawal syndrome (AWS), a serious and sometimes life-threatening event in alcohol-dependent individuals. The symptoms of AWS are profuse sweating, headache, nausea, vomiting, anxiety, tremor, tactile and auditory, as well as visual disturbances, agitation, disturbed orientation and clouding of sensorium (https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/alcohol-use-disorders). Acute AWS lasts up to 14 days after drinking is discontinued [3], and is most commonly measured by the Clinical Institute Withdrawal Assessment of Alcohol Scale, revised (CIWA-Ar), which assesses the symptoms using 10 items. The sum score of the items correlates with the severity of alcohol withdrawal [4].

Genetic factors play a role in the development of alcohol use disorder [5,6] as well as in the severity and time of onset of AWS [7–9]. Heritable components of AWS have been systematically explored by genome-wide association studies (GWAS) which have implicated several genes contributing to AWS, among which the sortilin family neurotrophin receptor gene (*SORCS2*) was observed repeatedly [10,11]. In addition to genetic factors, environmental factors play a role in the development of AUD and withdrawal responses. Environmental factors are thought to partially act via epigenetic changes of the genome, such as DNA methylation (DNAm), that can transiently or permanently change gene expression [12]. Alcohol consumption, a powerful environmental factor itself, might also lead to changes in DNAm. In recent years, DNAm alterations in AUD patients compared to controls have been identified in candidate genes such as *DAT* [13], *MAOA* [14], GABA receptor genes [15], *POMC* [16] and *SNCA* [17]. In general, it has been shown that global methylation levels change during alcohol detoxification [18]. Moreover, several epigenome-wide association studies (EWAS) of AUD have been reported. One study comparing DNAm signatures of 10 patients with alcohol use disorder and 10 healthy siblings detected 1581 differentially methylated cytosine–phosphate–guanine (CpG) sites [19]. A meta-analysis of EWAS in 13 317 individuals from 13 cohorts with European ancestry found that 363 CpG sites were associated with any alcohol consumption (at $P < 1 \times 10^{-7}$) [15]. In a cross-tissue and cross-phenotypical approach, Lohoff *et al.* integrated EWAS data from brain tissue and blood of AUD patients and controls and identified one gene, *PCSK9*, associated with disease phenotypes [20]. Furthermore, the impact of recent alcohol use on DNAm signatures was studied retrospectively in an EWAS by Philibert *et al.* [21] in a sample of 165 female individuals. They show that the degree of DNAm diminishes after a 30-day in-patient treatment program. Another EWAS further included a comparison of DNAm changes in 24 patients prior to and after a 3-week detoxification program and 23 controls [22]. At a DNAm difference of > 5%, 59 CpG sites were differentially methylated between patients and controls before entering the treatment program and 48 CpG sites were differentially methylated comparing patients before and after treatment.

Investigation of differentially methylated sites during withdrawal and comparisons to controls enables the identification of genes and pathways involved in withdrawal processes. This, in turn, may hint at relevant mechanisms underlying withdrawal and therefore improve the molecular understanding of this pathophysiological state. Once identified, epigenetic signatures may also be used as biomarkers for exposure or course of disease. In the present study, we investigated epigenome-wide methylation patterns in a longitudinal study of 99 severely alcohol-dependent patients during alcohol withdrawal (time-point 1) and after 2 weeks of recovery (time-point 2) to analyze epigenetic patterns specific to the state of withdrawal. We also compared the patients' methylation at both time-points to that of 95 age-matched controls to analyze enduring epigenetic patterns of AUD. A special focus of our study was to identify if the genes that were differentially methylated in patients compared to controls at time-point 1 showed a methylation pattern more similar to that of controls at time-point 2 (i.e. 'reversibility' of DNAm changes specific to alcohol consumption). Implicated molecular mechanisms were investigated using pathway analytical methods.

METHODS

Sample description

The sample consisted of male alcohol-dependent and age-matched male healthy control individuals (n = 100 each) recruited in the Department of Addictive Behaviour and Addiction Medicine at the Central Institute of Mental Health (CIMH) in Mannheim, Germany and in the addiction ward of the Ludwig Maximilian University (LMU) hospital for psychiatry and psychotherapy in Munich, Germany. Study procedures were carried out in accordance with the Declaration of Helsinki and were approved by the respective ethical committees of the study centers. All participants provided written informed consent.

All subjects were aged between 18 and 70 years. All patients were admitted to a medically supervised withdrawal treatment program. Healthy controls were recruited via newspaper announcements and through on-line information on the CIMH homepage.

For patients, inclusion criteria were presence of alcohol dependence syndrome according to DSM-IV criteria and presence of alcohol withdrawal syndrome as defined according to the CIWA-Ar. Patients had to present with a CIWA-Ar count of > 4 to be included in the present study. For LMU patients, withdrawal symptoms were assessed using the Alcohol Withdrawal Scale and converted to CIWA-Ar scores. Exclusion criteria were other severe mental illness (besides alcohol and nicotine dependence) that needed pharmacological treatment, severe physical illness that did not allow study participation, failure to provide written informed consent, other dependence syndromes according to the DSM-IV (except alcohol and nicotine dependence) and known neurological disorders. For healthy controls, exclusion criteria beyond those stated above were: any actual or past mental disorder (except nicotine dependence) assessed by SCID, risky drinking (> 60 g/48 g per day for men/women) on a regular basis, as assessed by Form90 interview [23] and positive drug urine screening. In patients, blood sampling was conducted on the first working day following admission and repeated 14 days after admission. In healthy controls, blood sampling was conducted on a working day within 7 days of screening. CIWA-Ar scores were assessed every 2 hours starting at the time of admission to the clinic for as long as needed to reach a stable subclinical value (2–4 days, on average).

Methylation measurement

DNA was extracted from full blood using the Chemagic Magnetic Separation Module I (Chemagen Biopolymer-Technologie AG, Baesweiler, Germany). All genomic DNA samples were stored at −20°C. DNA samples were carefully randomized on processing plates, arrays and positions on arrays and plates for measurement. To minimize batch effects across time, all samples obtained from the same individual were put onto the same array. Epigenome-wide methylation profiles were determined using Illumina EPIC methylation arrays and Illumina HiScan array scanning systems (Illumina, San Diego, CA, USA).

Data preprocessing, quality control and filtering

Data processing and analysis steps were performed using the R version 3.4.4 statistical analysis software (https://cran.r-project.org) and the R packages named below, unless otherwise mentioned. Methylation data were extracted from raw intensity data (idat files) using an updated version of the CPACOR pipeline published by Lehne *et al.* [24].

Samples with insufficient DNA quality (missing rate-0.05) or discrepancy between methylation-based and nominal sex were removed. Cross-hybridizing probes with an insufficient call rate (CR < 0.98), probes with SNPs located within the probe sequence [25] with a minor allele frequency (MAF) > 0.01 and sites located on either X or Y chromosomes were removed prior to analysis.

Data transformation, batch correction and cell type adjustment

Prior to association testing, methylation values were logit-transformed (base 2), and the resulting M-values were used as the dependent variable for association testing as recommended [26].

Technical quality and batch parameters were taken care of by extracting signals of internal control probes present on the Illumina EPIC array. Principal component analysis (PCA) was subsequently performed on these signals, and the leading 30 principal components resulting from this analysis were extracted as recommended [24] and included as covariates in all association tests.

In addition, white blood cell fractions were estimated using the method of Houseman *et al.* [27] as implemented in the minfi package [28], and the first five estimates (CD4T, CD8T, B cells, monocytes, natural killer cells) were then included in regression models to take confounding changes in cell type distribution into account. The last of the Houseman estimators (granulocytes) was skipped to avoid collinearity issues [variance inflation factor (within set of cell type estimates) VIF = 163].

Statistical analysis

Epigenome-wide methylation analysis. Tests of methylation differences between patients with acute withdrawal after 2 weeks of recovery, and controls were performed using a multiple linear regression approach as implemented in the limma package [28]. This method resembles an empirical Bayes extension to classical linear modelling that is more robust in the presence of outliers and hidden covariates [29].

As stated above, in addition to methylation M-values as the variable of interest, Houseman cell count estimates and 30 PCs from PCA performed on control probe signals were included as covariates to take confounding effects into account. Further possible confounding covariates included in the model were smoking and age, as these were reported previously to have a strong impact on methylation signals.

Within-patients comparison of methylation levels during acute withdrawal versus 2 weeks later was performed using linear mixed models, including individual ID as blocking factor.

Gene ontology (GO) over-representation analysis. GO analysis was carried out using the missMethyl version 1.12.0 package [30]. This analysis includes correction for selection bias arising due to different sizes of included genes/gene sets. The analysis included only sites obtaining FDR < 0.05 for case–control analyses and FDR $< 1 \times 10^{-4}$ for the longitudinal analysis, respectively, in the single marker test. GO categories obtaining FDR < 0.05 were

extracted and subsequently fed into http://amigo.geneontology.org/visualize?mode=client_amigo to visualize the networks.

Regional analysis. Chromosomal positions of CpG sites were annotated based on the manufacturers manifest file (ftp://webdata2:webdata2@ussd-ftp.illumina.com/downloads/productfiles/methylationEPIC/infinium-methylationepic-v-1-0-b4-manifest-file-csv.zip). Identification of differentially methylated regions was then performed using the comb-p program [31]. This method accounts for autocorrelation between tests of adjacent methylation sites, combines the respective sites to regions of enrichment and adjusts for multiple testing.

Pyrosequencing. The top-ranking CpG site in the DMR of the *TRIM39* gene was replicated by pyrosequencing (for details see Supporting information).

GWAS enrichment analysis. Multi-marker Analysis of GenoMic Annotation (MAGMA) [32] was used to integrate methylation signals with previously reported GWAS findings for alcohol dependence [33] and publicly available GWAS results for alcohol consumption from the UK Biobank (http://www.nealelab.is/uk-biobank). To do so, we created a gene set defined by genes harboring CpG sites that robustly differentiate between patients and controls at both time-points and tested whether significant findings from the above-mentioned alcohol dependence GWAS were enriched in the newly defined gene set. A second gene set was created based on sites that were significantly altered during acute withdrawal but reverted to normal levels afterwards to test for the involvement of genes associated with alcohol consumption in methylation changes associated with withdrawal.

Gene annotation for results tables. Genes were annotated to CpG sites using NCBI RefSeq genes, a curated subset annotation from the University of California Santa Cruz (UCSC) database (http://hgdownload.soe.ucsc.edu/goldenPath/hg19/database/). A CpG site was assigned to a gene if contained in its transcript region or up to 1500 base pairs upstream.

Pre-registration

The analysis was not pre-registered. Thus, the results should be considered exploratory.

RESULTS

Epigenome-wide methylation analysis

The final sample after quality control comprised 99 patients, each with blood samples drawn at two time-points —during an acute state of withdrawal and after 2 weeks of recovery—and 95 controls matched for sex (all males) and age. After filtering, 710 944 CpG sites present on Illumina EPIC array were available for single-site analysis.

Smoking status was highly correlated with AUD case status. For details about demographic characteristics please see Table 1.

Single-site analysis adjusted for technical quality parameters, age, cell count distribution and smoking revealed 2876 differentially methylated CpG sites (2517 up-, 359 down-regulated) comparing patients in acute withdrawal state and after 2 weeks of recovery, 9845 differentially methylated CpG sites (5847 up-, 3.998 down-regulated) in patients at time-point 1 when compared with controls and 6094 differentially methylated CpG sites at time-point 2 (3285 up-, 2809 down-regulated).

Association signals across chromosomal location are depicted in detail in Fig. 1 (Manhattan plots), and contrasted against effects size (mean methylation difference in %) in Supporting information, Figs S1–S3 (volcano plots). A list of top differentially methylated CpG sites in the longitudinal patient comparison is depicted in Table 2. The top signal in the longitudinal comparison is located in the *SCAP* gene on chromosome 3. For detailed information on all significant CpG sites (FDR < 0.05) in all three comparisons please see Supporting information, Tables S1–S3. The comparison of differentially methylated sites between cases and controls during acute withdrawal and after 2 weeks of recovery demonstrated that methylation levels of more than 841 CpG sites that had been significantly different to those of controls during acute withdrawal were

Table 1 Demographic data.

	Cases	*Controls*	*P-value (group comparison)*
Group size	99	95	0.77[a]
Age mean (SD)	47.6 (9.1)	47.4 (8.9)	0.89[b]
Smokers %	80	19	5.7×10^{-17}[a]
Cigarettes/day (in smokers) mean (SD)	25.8 (12.9)	18.7 (11.9)	0.05[b]
Family history of AUD[c] %	46	12	1.6×10^{-5}[a]
CIWA-Ar mean (SD)	9.7 (3.7)	NA[d]	NA[d]

[a]χ^2 test; [b]*t*-test; [c]First-grade relatives (in Munich subsample only refers to parents of participants); [d]not applicable. AUD = alcohol use disorder; CIWA-Ar = Clinical Institute Withdrawal Assessment of Alcohol Scale, Revised; SD = standard deviation.

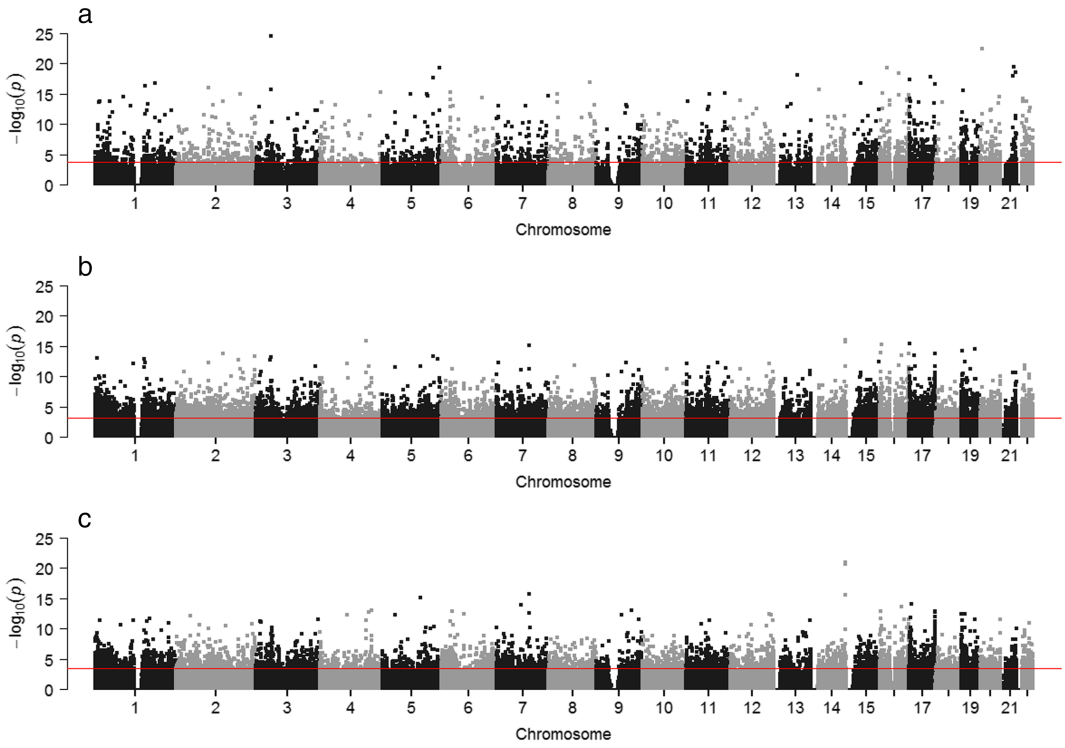

Figure 1 (a) Manhattan plot of association of methylation values with time-point in patients; (b) Manhattan plot of association of methylation values with case status at time-point 1; (c) Manhattan plot of association of methylation values with case status at time-point 2

Table 2 Results of pre–post comparison within patients (top 20 differentially methylated positions); all 20 sites were hypermethylated at time-point 1 (TP1) compared to TP2.

CpG	*Chromosome*	*Base pair position*	*Mean methylation difference, %*	*Average methylation, %*	*Nominal P-value*	*FDR*[a]	*Annotated genes*
cg21581415	3	47460784	5.1	62.7	2.8×10^{-25}	2.0×10^{-19}	*SCAP*
cg04937481	20	5637035	5.0	62.9	4.3×10^{-23}	1.5×10^{-17}	
cg03042032	21	39601516	4.8	52.4	3.7×10^{-20}	6.9×10^{-15}	*KCNJ15*
cg02481950	16	21665002	2.6	77.1	4.6×10^{-20}	6.9×10^{-15}	*METTL9*; *IGSF6*
cg18034719	5	176860863	2.7	84.9	4.9×10^{-20}	6.9×10^{-15}	*GRK6*
cg24707889	21	46341304	5.4	62.5	3.1×10^{-19}	3.7×10^{-14}	*ITGB22*; *ITGB2-AS1*
cg09674223	16	58914345	4.8	63.9	5.0×10^{-19}	5.1×10^{-14}	
cg01427460	13	72236233	4.4	71.5	7.3×10^{-19}	6.5×10^{-14}	*DACH1*
cg03288429	21	37732411	2.1	89.2	1.1×10^{-18}	9.0×10^{-14}	*MORC3*
cg17354333	17	66437754	3.4	73.9	1.7×10^{-18}	1.2×10^{-13}	*PRKAR1A*; *WIPI1*
cg19730422	5	156718560	5.0	59.1	2.1×10^{-18}	1.3×10^{-13}	*CYFIP2*
cg01291392	17	2264969	4.3	66.0	5.0×10^{-18}	3.0×10^{-13}	*SGSM2*
cg25428009	8	126304221	4.3	70.9	1.3×10^{-17}	7.0×10^{-13}	*NSMCE2*
cg13827596	1	185298424	6.1	59.8	2.0×10^{-17}	1.0×10^{-12}	*GS1-279B7.1*
cg03881200	15	44843088	2.6	53.8	2.2×10^{-17}	1.0×10^{-12}	*EIF3J*
cg26250129	17	79239903	4.3	66.1	2.3×10^{-17}	1.0×10^{-12}	*SLC38A10*
cg21961721	1	153751330	3.3	77.8	5.4×10^{-17}	2.3×10^{-12}	*SLC27A3*
cg25417988	2	99280314	4.7	49.8	1.1×10^{-16}	4.5×10^{-12}	*MGAT4A*
cg14335417	14	23393478	3.0	54.2	1.9×10^{-16}	7.2×10^{-12}	*PRMT5*
cg20366862	3	47098794	4.1	61.5	2.3×10^{-16}	8.0×10^{-12}	*SETD2*

[a]False discovery rate; CpG = cytosine–phosphate–guanine (CpG).

no longer significantly different compared to controls (Supporting information, Table S4).

Regional methylation analysis

Analysis using the 'comb-p pipeline' command (parameters seed = 10^{-5} and gap = 500, otherwise default) obtained 334, 438 and 307 significantly differentially methylated regions (DMRs) for the comparisons of time-point 1 versus time-point 2 within patients, patients at time-point 1 versus controls and patients at time-point 2 versus controls, respectively (please see Supporting information, Tables S5–S7 for the detailed results of the comparisons).

The top DMR in the longitudinal analysis is a region in the *TRIM39* gene within the major histocompatibility complex (MHC) region on chromosome 6 (see Fig. 2). The top-ranking CpG site in this DMR was validated by pyrosequencing.

GWAS enrichment analysis

There was no significant enrichment of GWA signals for alcohol dependence in sites robustly differentiating between patients and controls.

A gene set analysis based on the abovementioned 841 sites with reversible methylation changes during acute withdrawal did not obtain a significant enrichment of GWA signals for alcohol consumption from the UK Biobank results.

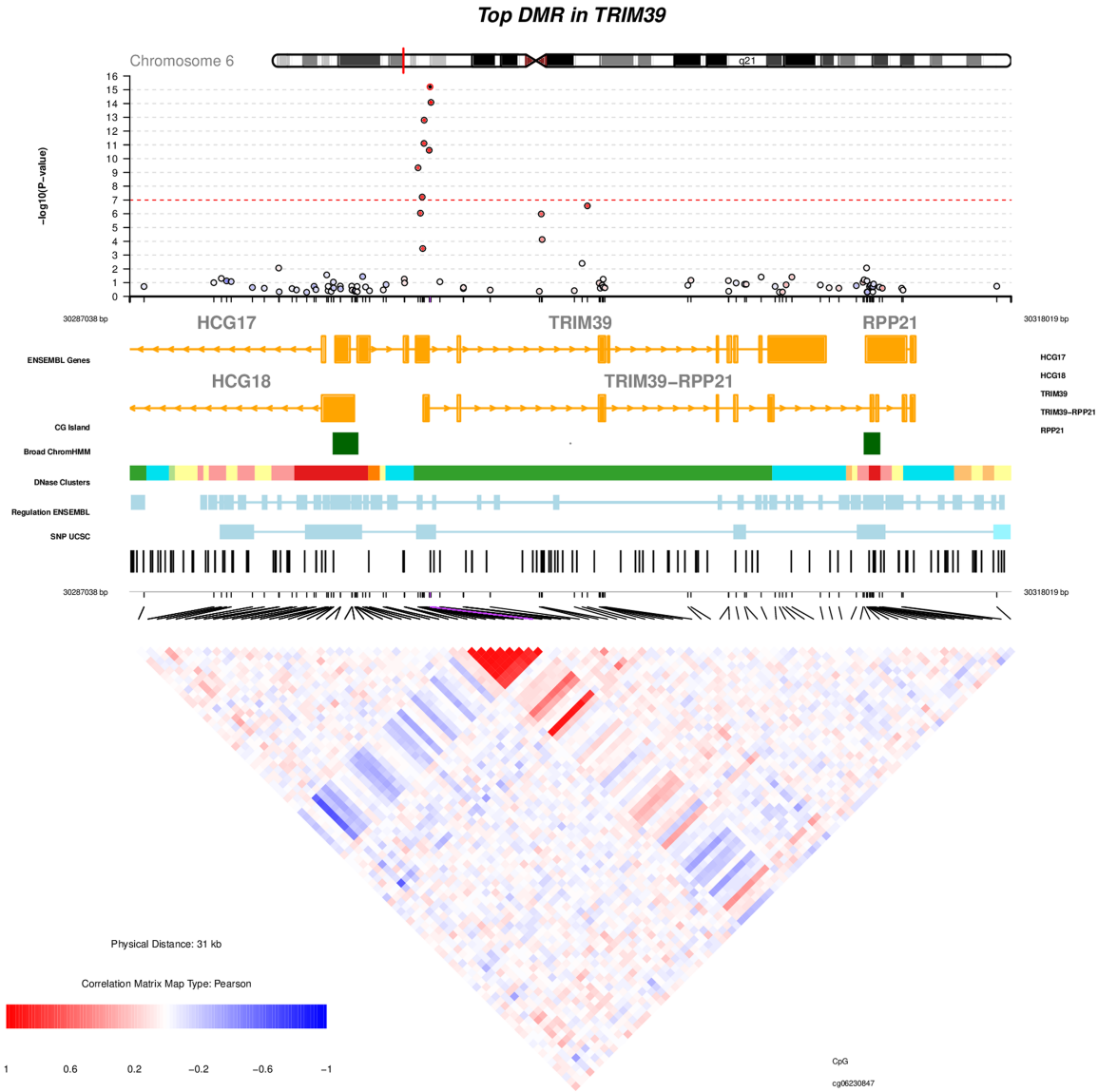

Figure 2 Regional association plot showing TRIM39 *P*-values in longitudinal analysis, including pairwise correlation between adjacent sites and additional annotation tracks

Gene ontology over-representation analysis

Analysis of over-representation of associated genes in GO terms revealed a significant over-representation of 15 GO terms in the comparison of patients in acute withdrawal and after 2 weeks of recovery, and of 61 and 10 GO terms in the comparisons of patients at time-point 1 versus controls and patients at time-point 2 versus controls, respectively, after FDR correction. Descriptively, in the comparisons of patients at time-point 1 and control as well as patients at time-point 1 versus time-point 2, GO terms annotating immunological and inflammatory system processes were noticeably over-represented. Interestingly, the comparison patients at time-point 2 versus controls did not yield an over-representation of these processes. All but one GO term in this comparison also resulted in the comparison of patients at time-point 1 versus controls.

For details of GO over-representation analysis please see Supporting information, Tables S8–S10.

DISCUSSION

In the present study, we report the largest analysis of epigenome-wide methylation patterns longitudinally investigated in 99 severely alcohol-dependent male patients during alcohol withdrawal (time-point 1) and after 2 weeks of recovery (time-point 2). Methylation patterns of patients at both time-points were also compared to methylation patterns of 95 age-matched male controls.

We found significant and widespread changes in DNA methylation in the longitudinal comparison of patients as well as between patients and controls. A large number of CpG sites were differentially methylated during withdrawal and after 2 weeks of recovery within patients (2876 CpG sites), among them CpG sites in genes reported to play a role in withdrawal symptomatology in previous studies (e.g. *SLC29A1*, *FYN*) [34,35]. As expected, methylation between patients and controls also differed considerably (9845 and 6094 CpG sites comparing patients at time-points 1 and 2 versus controls, respectively) at CpG sites in genes that had previously been implicated in withdrawal (e.g. *FKBP5*, *BDNF*, *EFNA5*) [10,36,37]. Apart from genes that have already been implicated in withdrawal, we found a large number of genes whose role in withdrawal is still unclear. For example, the top hit of our longitudinal comparison is *SCAP*. SCAP is an escort protein required for cholesterol synthesis as well as lipid homeostasis (https://www.genecards.org/cgi-bin/carddisp.pl?gene=SCAP). In a previous network analysis of alcohol consumption and withdrawal, *SCAP* was among the differentially expressed genes [38].

The high number of differentially methylated CpG sites in AUD patients compared to controls is consistent with the literature [15,20]. There may be several reasons for this: DNAm has a strong genetic underpinning [39–41], and thus methylation patterns may be different for individuals who become alcohol-addicted in contrast to individuals who stay healthy. Additionally, alcohol is a potent agent, and the observed differences between patients and controls may demonstrate the strong effect of alcohol consumption, an effect which is reduced after cessation of drinking but continues to be observable long afterwards [42]. In particular, our results are in line with studies showing pronounced differences between patients in acute withdrawal and after 2 weeks of recovery [22,43].

The number of differentially methylated CpG sites in patients compared to controls decreases after 2 weeks of recovery. Also, after 2 weeks of recovery, methylation levels of more than 800 CpG sites that had been significantly different to those of controls during acute withdrawal were no longer significantly different in patients compared to controls. This suggests that methylation levels in patients approach normal levels after withdrawal, mirroring phenotypical recovery, which indicates a reversibility of withdrawal-specific DNAm. This replicates findings from other studies demonstrating that DNAm reverts to normal after cessation of alcohol consumption [22,43].

Accounting for the fact that single CpG sites are not independent of one another, we furthermore examined differentially methylated regions (DMRs) of CpG site clusters in which methylation varied within patients during acute withdrawal and after 2 weeks of recovery and patients compared to controls at the two time-points. We found significant DMRs in all three comparisons. The top DMR in the longitudinal analysis is in the tripartite motif-containing 39 gene (*TRIM39*) located in the major histocompatibility complex (MHC) class I region on chromosome 6. *TRIM39* functions as the E3 ubiquitin ligase and is involved in inflammatory processes by negatively regulating the nuclear factor kappa B (NF-κB) signal induced by inflammatory stimulants such as tumor necrosis factor (TNF)-α [44]. The top associated CpG sites in this region were validated using pyrosequencing.

To identify molecular functions inherent to the differentially methylated genes on a more global level, we performed a network analysis searching for over-representation of differentially methylated genes in GO terms. Our findings suggested that pathways involved in immunological and inflammatory responses are strongly affected during withdrawal. This was also the result in the comparison of patients at time-points 1 and 2. Interestingly, the comparison of patients at time-point 2 versus controls did not find an over-representation of GO terms involved in immunological and inflammatory response.

These findings are of interest in light of previous studies linking immune function to alcohol consumption and

alcohol withdrawal: chronic exposure to alcohol leads to multiple organ damage, including damage to the adaptive as well as innate immune systems, which exhibit accelerated inflammatory responses [45–47]. An activated immune response has been shown on the protein level [48,49] as well as on the transcription level [50] during alcohol consumption and withdrawal while, during abstinence, the immune response reverts to normal [48,49]. Our results show that the changes of the immune response as well as their reversibility after withdrawal is reflected in underlying changes on the methylation level.

Other pathways remain different between patients and controls after withdrawal. These might represent pathways implicated in alcohol addiction itself or the vulnerability to it. For example, two pathways refer to the regulation of GTPase activity (GO:0043087 and GO:0043547). Rho-type GTPases have been implicated in the regulation of behavioral responses to ethanol exposure in *Drosophila melanogaster* [51]. Moreover, Ras suppressor 1 (Rsu1), which links signaling from the integrin cell adhesion molecule to the GTPase Rac1 in adult neurons, regulates reward-related behaviors such as ethanol consumption [52].

As recent GWAS have demonstrated that SNPs associated with complex disorders are enriched in epigenetic regions, we analyzed the enrichment of genome-wide significant GWAS signals for alcohol dependence in CpG sites that were able to robustly differentiate between patients and controls as well as the enrichment of genome-wide significant GWAS signals for alcohol consumption in CpG sites with reversible methylation changes during acute withdrawal. We did not find a significant enrichment for either gene set.

This study has several limitations. A major limitation of the present study is that we could not control for the influence of medication on methylation levels, due to missing information. Thus, the possibility that medication might have influenced our results cannot be excluded, although Koller and colleagues recently demonstrated that potentially influencing variables, such as dose of withdrawal medication, does not significantly influence epigenetic changes [18]. In any case, prior evidence from the literature provides independent support for our finding: the effect of alcohol consumption on the immunological and inflammatory response and its reversibility have been demonstrated in numerous studies, although it cannot be definitively stated whether the immune response is due to alcohol consumption, withdrawal or both [46,48–50,53].

Moreover, due to lack of data, we cannot disentangle the effects of withdrawal from those of accompanying changes in the environment, possibly related to the recovery period such as improved diet, decrease in overall stress associated with previous drug-seeking during heavy drinking periods, changes in sleep patterns or smoking cessation. With respect to the latter, however, we did not find any evidence in the methylation patterns that would indicate that subjects had quit smoking.

Another limitation is that methylation was analyzed in peripheral tissue, which does not entirely reflect methylation changes in the brain [54]. However, many of the genes that were differentially methylated are also found in the brain [55]. Furthermore, previous reports have demonstrated the value of analyzing peripheral tissue to explore molecular mechanisms and detect new genes, pathways and biomarkers for psychiatric diseases [56,57]. These potential biomarkers must be measurable in easily accessible tissue if they are to be developed for routine clinical use.

In the largest study to date comparing DNAm in patients during acute withdrawal and after 2 weeks of recovery, we replicate known genes and suggest novel genes which may play a crucial role in alcohol withdrawal. Further studies will have to corroborate these novel genes and elucidate their function within AUD and withdrawal. We also emphasize the close relationship between alcohol consumption and the immune response for the first time at the methylation level, and demonstrate that after cessation of alcohol drinking, methylation of immune response genes reverts back to normal. Closer investigation of these systems with larger sample sizes and denser, longer-term sampling schemes will be key to disentangling and understanding the multi-level interactions at work.

Declaration of interests

None.

Acknowledgements

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1 Supporting Information.

Figure S1 Volcano plot contrasting effect size vs p-Value of association of methylation values with time point in patients.

Figure S2 Volcano plot contrasting effect size vs p-Value of association of methylation values with case status at time point 1.

Figure S3 Volcano plot contrasting effect size vs p-Value of association of methylation values with case status at time point 2.

Table S1 CpG sites significantly associated with withdrawal state in patients after FDR correction.
Table S2 CpG sites significantly associated with case control status at time point 1 after FDR correction.
Table S3 CpG sites significantly associated with case control status at time point 2 after FDR correction.

Table S4 CpG sites significantly associated with case control status at time point 1 but not time point 2 after FDR correction.

Table S5 DMRs significantly associated with withdrawal state in patients after FDR correction.

Table S6 DMRs significantly associated with case control status at time point 1 after FDR correction.

Table S7 DMRs significantly associated with case control status at time point 2 after FDR correction.

Table S8 Overrepresented GO terms in longitudinal comparison of patients after FDR correction.

Table S9 Overrepresented GO terms in comparison of patients at time point 1 versus controls after FDR correction.

Tables S10 Overrepresented GO terms in comparison of patients at time point 2 versus controls after FDR correction.