



Published in final edited form as:

Alcohol Clin Exp Res. 2019 October ; 43(10): 2111–2121. doi:10.1111/acer.14168.

Epigenome-wide DNA Methylation Association Analysis Identified Novel Loci in Peripheral Cells for Alcohol Consumption among European American Male Veterans

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Abstract

Background: Hazardous alcohol consumption has significant adverse medical consequences. These effects may be mediated, in part, by alterations in DNA methylation. Thus, DNA methylation signatures in peripheral cells may provide biomarkers of the medical impact of alcohol use and the risk for future alcohol consumption.

Method: Using a high density methylation array, we characterized epigenome-wide DNA methylation in saliva cells with respect to alcohol consumption in a large cohort of male European American veterans. In this study, DNA methylation of over 870,000 CpG DNA sites was profiled in 1,135 European American men. Alcohol consumption was assessed using the Alcohol Use Disorder Identification Test-Consumption (AUDIT-C). Linear regression was applied in an epigenome-wide association study (EWAS), adjusted for confounders. Gene set enrichment analysis was performed in the KEGG database with a correction for gene length.

Results: We found that a total of 70 CpG sites reached EWAS-corrected significance ($p < 6E-08$) with small effects on alcohol consumption for individual CpG sites, including 64 new CpG sites and six CpG sites that were previously reported as associated with alcohol use disorder, liver function, body mass index, and lipid metabolism. The most significant CpG site was located in *SLC7A11* ($t = -11.34$, $p = 2.66E-28$), a gene involved specifically in cysteine and glutamate transportation. The 70 significant CpG sites were located on 44 genes, including genes involved in amino acid transport and metabolism systems. We identified 68 pathways with a false discovery rate < 0.05 .

Conclusion: We identified novel DNA methylation sites associated with alcohol consumption. Results may shed light on peripheral mechanisms of alcohol consumption on adverse health outcomes among heavy drinkers.

Keywords

Alcohol consumption; DNA methylation; Epigenome-wide Association Study (EWAS)

Introduction

Alcohol consumption is a significant risk factor for psychiatric and medical diseases and is the seventh leading cause of death and disability worldwide (Burton and Sheron, 2018, Collaborators, 2018). Many of the mechanisms whereby alcohol consumption exerts adverse effects on health outcomes are unclear. Emerging evidence suggests that high levels of alcohol consumption alters epigenetic markers in both the central nervous system and peripheral tissues (Liu et al., 2018, Lussier et al., 2018, Berkel and Pandey, 2017), suggesting a role of epigenetic regulation consequent to alcohol exposure that may contribute to alcohol-related health outcomes.

DNA methylation (DNAm), the most commonly studied epigenetic mechanism, involves a process in which a methyl group is added to the cytosine pyrimidine without changing DNA sequence. DNAm is modulated by DNA methyltransferase which transfers a methyl group from S-adenosyl methionine (SAM) to the 5-position of cytosine in the context of cytosine-phosphate-guanine (CpG) dinucleotide. Hazardous alcohol consumption often causes folate and vitamin B deficiency; these are cofactors in methyl transfer reactions, and deficiency reduces the level of SAM (Williams et al., 1949). Thus, it has been hypothesized that DNAm alterations by hazardous alcohol consumption plays a critical role in regulating gene function (Berkel and Pandey, 2017, Palmisano and Pandey, 2017, Tulisak et al., 2017).

In animals, decreased methylase activity and DNA hypomethylation of CpG sites were found in ethanol-treated developing brains (Garro et al., 1991). DNAm signatures at the nucleus accumbens core of male rhesus macaques reportedly differentiated light and heavy alcohol consumption (Cervera-Juanes et al., 2017). Alcohol exposure resulting in DNAm perturbation in developing brain can persist into adulthood in mice (Zhang et al., 2015). In humans, due to the limitation of access to living human brain, the majority of DNAm studies for the purpose of identifying biomarkers for alcohol consumption, as well as for guiding treatment have been conducted in peripheral cells (Lussier et al., 2018, Liu et al., 2018). DNAm of candidate genes that previously identified for a risk of Alcohol Use Disorder (AUD) through genetic association studies have been reported in relation to AUD and fetal alcohol spectrum disorder diagnoses, symptom severity, alcohol withdrawal, and treatment response (Zhang and Gelernter, 2017, Bruckmann et al., 2016, Heberlein et al., 2013, Hillemaier et al., 2015, van der Knaap et al., 2014, Laufer et al., 2015, Portales-Casamar et al., 2016). However, these studies were conducted in small samples and the findings were inconsistent. Recently, epigenome-wide association studies (EWAS) of DNAm in peripheral blood have identified widespread DNAm changes in the epigenome in individuals with hazardous alcohol use or AUD. The majority of the reported DNAm loci were found to be hypomethylated in individuals with AUD relative to those without AUD (Philibert et al., 2014, Bruckmann et al., 2017). The largest meta-EWAS to date in 13,317 individuals – a rather heterogeneous sample -- identified hundreds of DNAm sites. A selected set of

144 CpGs enabled differentiation of alcohol drinking and non-alcohol drinking subjects, suggesting that DNAm sites could serve as biomarkers for alcohol consumption(Liu et al., 2018). A study conducted in isolated CD3+ T cells revealed 48 CpG sites associated with AUD treatment response(Bruckmann et al., 2017).

A notable limitation of DNAm candidate gene studies is that DNAm loci identified in such studies are rarely replicated in EWAS(Zhang and Gelernter, 2017). The findings of DNAm for AUD are also inconsistent among the reported EWAS to date, which may be due to either lack of power(Philibert et al., 2014, Hagerty et al., 2016), lack of correction of cell type effects on DNAm (Hagerty et al., 2016), using relatively low density of CpG array in epigenome(Zhang et al., 2013), and the sample heterogeneity expected to result from meta-analysis. Here, we report an EWAS for alcohol consumption in a comparatively large sample of U.S. veterans (N= 1,135) using a high density methylation array with 850,000 CpG sites in the epigenome. Because of the available source of DNA, the study aimed to understand peripheral mechanisms of alcohol consumption rather than exploring the neural mechanism of AUD, except to the extent that peripheral measures reflect central activity. We focused on European Americans to limit the confounding effects of ethnicity. Because the study population was predominantly male U.S. military veterans, we excluded women in our analysis to rule out sex effects on DNAm, lacking the power necessary to examine this important question.

Materials and methods

Study population

Our study sample included 1,135 European American (EA) male U.S. military veterans who participated in the National Health and Resilience in Veterans Study (NHRVS)(Stefanovics et al., 2018, Fuehrlein et al., 2018). The NHRVS is a nationally-representative study of U.S. veterans, where recruitment was conducted in October-December 2011. Participants were recruited from a research panel comprising more than 50,000 households developed and maintained by GfK Knowledge Networks, Inc. (Menlo Park, CA, USA). All participants provided informed consent. Demographic and substance use characteristics are presented in Table 1. This study was approved by the Human Subjects Subcommittee of the Veterans Affairs (VA) Connecticut Healthcare System and VA Office of Research & Development.

Alcohol consumption

Alcohol consumption was assessed via self-report using the Alcohol Use Disorder Identification Test-Consumption (AUDIT-C)(Bush et al., 1998). Scores on the AUDIT-C range from 0–12. The mean score in our samples was 2 ± 2.4 and 28.7% (N=326) screened positive for hazardous alcohol consumption with a cutoff score ≥ 4 . The distribution of AUDIT-C scores is shown in Supplemental Figure S1.

DNA methylation quantification and quality control

Genomic DNA (500 ng) was extracted from saliva samples using Oragene kits (DNA Genotek, Ottawa, Ontario, Canada) and treated with bisulfite reagents included in the EZ-96 DNAm kit (Zymo Research, Orange, CA, USA) following the manufacturer's standard

protocol. Bisulfite-converted DNA samples were used in the array-based DNAm assay, the Illumina Infinium Human MethylationEPIC BeadChip (Illumina, San Diego, CA, USA), which interrogates DNAm at >850,000 loci genome-wide. DNAm profiling was conducted at the Yale Center for Genome Analysis. The data processing and analytical framework is presented in Figure S2.

Quality control was conducted in R version 3.4.1 and performed based on a pipeline using the ‘minfi’ R package (Bioconductor 1.8.9)(Aryee et al., 2014). To ensure that only high-confidence probes are included, probes with detection p -value > 0.001 were removed. We also removed probes: 1) with annotated single nucleotide polymorphisms (SNPs) at SBE/CpG sites (via the Single Nucleotide Polymorphism database 137 [National Center for Biotechnology Information]), 2) cross-hybridizing probes, and 3) sex chromosome probes. The Combat method in the ‘sva’ package(Leek et al., 2012) was applied to correct for batch effects to adjust for sample plate and cohort group. Data were normalized using the functional normalization method(Aryee et al., 2014) implemented in the ‘minfi’ R package, which uses internal control probes present on the array to control for between-array technical variation and known to outperform other existing approaches(Fortin et al., 2014). A total of 756,573 were left for subsequent analysis.

Cell type proportion in the peripheral saliva samples (e.g. CD34, CD14, and buccal cells) was estimated using a modified version of the Houseman and colleagues method(Houseman et al., 2012, Smith et al., 2015). To adjust for possible population stratification, a methylation-based principal component (PC) approach(Barfield et al., 2014) was conducted based on sets of CpG sites within 50 kb of SNPs using the 1000 Genomes Project variants with minor allele frequency (MAF) > 0.1(Barfield et al., 2014). The first 10 PCs were used in the analysis as covariates.

Statistical analysis

All statistical analyses were performed within R 3.4.0 (www.r-project.org). EWAS analysis was conducted using the ‘cpg.assoc’ function from the ‘minfi’ R package(Barfield et al., 2012), adjusted for age, current smoking status, and estimated cell proportions. We found no significant differences in estimated cell proportions between the non-hazardous and hazardous alcohol consumption groups (p s > 0.05). These covariates were nevertheless adjusted in the model as a “best practice” to correct for potential confounding effects. We also adjusted top the 10 PCs in the model to correct the effects of population admixture. The significance level was set at nominal $p=6E-08$, which is equivalent to Bonferroni correction for a significance level of $p=0.05$. Considering a possible close relationship between body weight and alcohol consumption, we did an additional analysis to test whether body mass index (BMI) confounded the effect of alcohol consumption on DNAm. (However note that in our sample, BMI was lower in the Hazardous alcohol consumption group than in the non-Hazardous alcohol consumption group – Table 1.) Further, we tested associations of EWAS-significant CpG sites with hazardous alcohol consumption that was defined with AUDIT-C score ≥ 4 . Methylation CpG sites associated with alcohol consumption at the $p<5E-04$ level (level chosen empirically to ensure a statistic power with a sufficient gene set) were selected for gene set enrichment analysis. Considering that gene length may lead to

bias of gene-set enrichment analysis as a result of different numbers of CpG sites in different genes (Geeleher et al., 2013), we adjusted the gene length by using missMethyl program in BioConductor (<http://bioconductor.org/packages/release/bioc/html/missMethyl.html>). False discovery rate (FDR) < 0.05 was a cutoff for statistical significance.

Results

Epigenome-wide association study for alcohol consumption

We identified 70 epigenome-wide significant (EWS) CpGs associated with AUDIT-C scores (Figure 1 and Table 2). Six of these 70 CpG sites were previously reported as associated with alcohol consumption or other alcohol-related phenotypes such as liver function and body weight, and 64 CpG sites were novel for alcohol consumption. Fifty-one out of 70 CpGs were located in a gene region: as expected, a majority of significant CpGs was in a gene body, while 16 CpGs were in promoter or UTR regions. Unlike previous reports showing that significantly more hypomethylation was associated with alcohol consumption (Liu et al., 2018, Hagerty et al., 2016), we found that methylation level was inversely associated with AUDIT-C scores at 37 CpG sites while methylation level was positively associated with AUDIT-C at 33 CpG sites.

Multiple CpG sites in promoter regions reached epigenome-wide significance. For example, cg16834011 in the 5'UTR of *COMT* was inversely associated with alcohol consumption ($t=-6.84$; $p=1.33E-11$), while cg16702725 in TSS1500 region of *PCP4L1* was positively associated with alcohol consumption ($t=6.07$; $p=1.76E-09$). Other CpGs in the promoter regions included cg01764256 in *ZMYND8*, cg00508575 in *ATP2B1*, cg06644515 in *SNORD80*, cg06739059 in *ITPRIPL1*, cg07626482 in *SLC1A5*, and cg11701312 in *RPS5*.

The most significant CpG, cg06690548, was located in the gene body of *SLC7A11* ($t=-11.35$, $p=2.66E-28$), and was inversely associated with AUDIT-C score. This gene encodes a member of a heteromeric, sodium-independent, anionic amino acid transport system that is highly specific for cysteine and glutamate. Another CpG site in a sodium transporter gene, *SLC1A5*, was also inversely associated with alcohol consumption (cg07626482, $t=-5.66$; $p=1.91E-08$) while CpG, cg16968128, located at in a sodium channel gene, *SLC34A1*, showed positive association with AUDIT-C scores ($t=5.85$, $p=6.31E-09$). Unlike *SLC7A11*, *SLC34A1* is involved in sodium-dependent phosphate transporter 2A (NaPi-IIa), which plays a role in the regulation of phosphate homeostasis.

Adjustment for BMI—Adding BMI into the analytic model, we found 58 out of 70 identified CpG sites remained EWS (Table 2). Twelve CpG sites were close to EWS with slightly reduced significance (p in a range of $2.67E-07$ ~ $1.70E-07$). Significant CpG sites on amino acid transporter genes (*SLC7A11*, *SLC1A5*, and *SLC34A1*) were still significantly associated with AUDIT-C. Taking into account the reduction in power consequent to adding an additional covariate, the results suggest that BMI is not a major confounding factor for EWAS on alcohol consumption in our cohort.

Differential DNAm between hazardous and non-hazardous alcohol consumption

We conducted a secondary analysis to estimate the relationship of EWAS-significant CpG sites for alcohol consumption to hazardous alcohol drinking using an AUDIT-C score ≥ 4 as a cutoff. We found 12 out of 70 CpG sites implicated in the primary analysis, also differentially methylated between hazardous and non-hazardous alcohol consumption with Bonferroni $p < 0.05$. The difference of DNAm at single CpG sites between hazardous and non-hazardous alcohol consumption was small across 12 significant DNAm loci. The difference of methylation β at each CpG site between hazardous and non-hazardous alcohol consumption was less than 5%. The largest differential DNAm CpG was 4% at cg06690548 in *SLC7A11* (Supplemental Table S1). Figure 2 presents methylation level of 12 significant CpGs of DNAm between hazardous and non-hazardous alcohol consumption.

Post-EWAS analysis and biological interpretation

A total of 729 CpG sites with $p < 5E-04$ were used for gene set enrichment analysis for alcohol consumption based on the sum of AUDIT-C score, not on hazardous use. These 729 CPG sites mapped to 527 genes. We found 68 significant pathways enriched in the KEGG database (Supplemental Table S2). Figure 3 presents the top 10 pathways associated with alcohol consumption. All significant pathways are presented in Table S2. Metabolic pathways was the most significant pathway associated with alcohol consumption (FDR=1.58E-08). Several pathways were relevant to cancer development, e.g. MicroRNAs in Cancer (FDR=5.96E-04), Proteoglycans in cancer (FDR=6.686E-04), Pathways in cancer (FDR=1.192E-04), Transcriptional misregulation in cancer (FDR=1.97E-05), and Pancreatic cancer (FDR=1.868E-02). Pathways involving viral or bacterial infection pathogenesis were also significant, e.g. Human cytomegalovirus infection (FDR=6.77E-05). Signaling pathways such as MAPK signaling pathway (FDR=5.96E-04), and Rap1 signaling, pathway (FDR=2.602E-04), were also statistically significant.

Discussion

Using a high-density DNAm array, we completed an EWAS for alcohol consumption in a large EA male veteran population. We identified 64 novel differentially methylated loci in peripheral cells and replicated three previously reported loci associated with alcohol consumption (Liu et al., 2018) and three loci previously associated with alcohol-related medical conditions such as liver function (Nano et al., 2017) and body mass index (BMI) (Aslibekyan et al., 2015) (but not previously associated to alcohol use *per se*). Alcohol consumption-associated loci were located in genes involved in amino acid transport, synthesis, and metallization, and with important roles in cellular processes, development, and apoptosis. The alcohol-related genes identified from this EWAS were enriched in biological pathways related to cellular metabolism, infection, cancer, and signaling transduction. Because alcohol consumption is known to increase risks of cancer (Seitz et al., 2018, Bagnardi et al., 2013), infectious diseases (Trejejo-Nunez et al., 2015, Szabo, 1999), and metabolic syndrome (Freiberg et al., 2004), these findings suggest that the alteration of DNAm in peripheral cells associated with alcohol consumption may implicate DNAm in peripheral cells in alcohol-related medical outcomes.

It is important to consider the correlation of DNAm in saliva cells compared to other tissues. Emerging evidence suggests that a set of DNAm loci are highly correlated across different tissues including DNAm between blood and brain regions (Qi et al., 2018) and saliva and brain regions (Smith et al., 2015), suggesting that a proportion of DNAm sites in peripheral cells may be consistent with the methylation in neural cells. DNAm is correlated between saliva and intestinal mucosal cells within an individual; only approximately 11% of CpG sites are differentially methylated between saliva and intestinal mucosa (Hearn et al., 2019). A recent study reported 20 out of 26 CpG sites associated with smoking were consistent between blood and saliva cells (Barcelona et al., 2019). One interpretation of the highly correlated markers in different tissues is that pathophysiological processes resulting from environmental exposure and lifestyle (e.g. smoking, alcohol use) are involved in epigenetic alterations on a set of CpG sites across different tissues to regulate genes in response or counter response to such insults. Therefore, CpG sites identified in peripheral tissues such as blood and saliva could serve as surrogate markers for the phenotype of interest. This is especially useful for behavioral phenotypes considering the inaccessibility of live human brain. Study of peripheral DNAm has a very strong advantage of possible utility in identifying biomarkers, which, if identified in the brain, cannot be applied clinically.

We found that changes of DNAm associated with alcohol consumption were small: the DNAm differences between hazardous alcohol use and non-hazardous alcohol use were all <5%. These findings are consistent with expectation; unlike the effects of DNAm on oncogenes for cancers, emerging evidence has shown that intermediary DNAm level has become an epigenetic hallmark for many complex, non-malignant diseases and environmental stimuli (Leenen et al., 2016, Levenson, 2010). Differential DNAm between case and control groups of complex traits are typically <10% and often between 1–5% at single CpG sites. Some well-established methylation marks for hypertension, obesity, cardiovascular disease, major depression, and schizophrenia (Han et al., 2018, Ota et al., 2014) have shown small effects. Subtle DNAm changes associated with smoking and alcohol consumption have also been observed in previous studies (Gao et al., 2015, Harlaar and Hutchison, 2013, Liu et al., 2018). Although the underlying biology of mild DNAm alterations remains unaddressed, these methylation marks are often reliably detected across different phenotypic assessments, sample populations, and DNAm platforms. For example, several CpG sites in our study profiled by means of the Illumina EPIC platform were previously reported in studies using the Illumina 450K platform. Specifically, cg06690548 on *SLC7A11* ($p=2.66E-28$), cg16246545 on *PHGDH* ($p=4.95E-11$), cg06469895 on *TERF2* ($p=2.39E-10$), and cg02583484 on *HNRNPA1* ($p=4.83E-08$) were found to have significant associations alcohol consumption in our study and in Liu et al (Liu et al., 2018). Another four CpG sites, cg05993525 on *TRERFI* ($p=4.43E-07$), cg2706351 on *DENND2D* ($p=1.24E-06$), cg0352740 on *TXLNA* ($p=2.03E-06$), and cg21180953 on *SETBP1* ($p=3.03E-6$) were among the top significant genes associated with alcohol consumption in EAs(Liu et al., 2018) and, as noted, were of suggestive significance in our study. In a meta-analysis(Liu et al., 2018), p values for CpG sites on these four genes ranged from $3.2E-12$ to $4.4E-15$. Taken together, these data suggest that DNAm is fine-tuned in the epigenome for complex diseases and that pronounced regulation of gene transcription related to complex behaviors may generally not be expected. Instead,

subtle changes of DNAm associated with alcohol consumption may be associated to a wide range of variability for alcohol consumption for this highly prevalent trait in the general population. This is rather similar to the case of stable genetic SNP markers detected by Genome-wide association studies which, for complex traits, generally have small-to-very small effect sizes.

Of note, several genes identified in our EWAS are involved in amino acid transport and metabolism. For example, 3 CpGs, cg14476101, cg16246545, and cg26457483 on *PHGDH*, were hypomethylated in hazardous alcohol users compared to non-hazardous alcohol users in our sample (p values ranging 2.73E-08 – 3.17E-11). This gene encodes the enzyme involved in the first step of serine biosynthesis. D-serine is a co-agonist for the N-Methyl-D-aspartic acid glutamate receptor and exogenous D-serine has been showed to reduce alcohol consumption in animals (Seif et al., 2015). Serine was also demonstrated to ameliorate alcoholic fatty liver by accelerating serine-dependent homocysteine metabolism in mice and rats (Sim et al., 2015). Methylation of the same locus has been linked to BMI and waist circumference by EWAS (Wahl et al., 2017). These studies revealed that one of the three significant alcohol-related CpG sites on *PHGDH*, cg14476101, was also inversely associated with triglyceride levels (Truong et al., 2017) and liver enzymes (Nano et al., 2017).

The most significant CpG among these is cg06690548 on *SLC7A11* (p=2.66E-028), which was inversely associated with alcohol consumption in our sample. As mentioned early, *SLC7A11* is highly specific for cysteine and glutamate transportation. The cysteine/glutamate antiporter brings cysteine into the cell in exchange for glutamate exported from the cell. The cysteine brought into the cell is often used as a substrate for glutathione, a “free radical” scavenger that protects the cell from injury. Thus, increased transcription of this antiporter gene could be a signal of cellular response to redox threat, which may implicate a mechanism for increased cancer risk from alcohol consumption (Habib et al., 2015, Linher-Melville et al., 2015). Similar to cg06690548 on *SLC7A11*, two other significant CpGs mapped on the *SLCIA5* (cg07626482, t= -5.66; p=1.91E-08) and *SLC34A1* (cg16968128, t=5.85, p=6.31E-09), genes that are involved in amino acid transportation and biosynthesis that may involve mechanisms of cancer. For example, *SLCIA5* is a glutamate transporter reported to facilitate the uptake of glutamine in tumor cells, which in turn serves multiple metabolic functions within cells depending on the tumor oncogenotype and microenvironment (Altman et al., 2016). Methylation on these three genes, *SLC7A11*, *SLCIA5* and *SLC34A1*, is also linked to liver function, lipid metabolism, and BMI (Nano et al., 2017, Wahl et al., 2017).

Alcohol has many peripheral effects also, raising our confidence in these findings; and in fact the best-replicated genetic variants influencing alcohol use disorder risk, map to genes encoding alcohol metabolizing enzymes that can act peripherally (Walters et al., 2018, Edenberg, 2007). Notwithstanding the noted limitations, the results of this study reveal possible peripheral mechanisms associated with alcohol consumption. They also provide further evidence of alcohol exposure’s impact on the epigenome and its role in alcohol-related health problems. The identified DNAm signatures may thus potentially serve as biomarkers of alcohol consumption and risk for alcohol-related health problems. Further

research is needed to replicate these results in independent well-powered samples, as well as in more heterogeneous samples (e.g., women, different ethnic populations); and to evaluate the biological roles of the small effects of the identified CpG sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments and Disclosures

The National Health and Resilience in Veterans Study is supported by the U.S. Department of Veterans Affairs National Center for Posttraumatic Stress Disorder. This work was supported in part by National Institute on Drug Abuse (NIDA, R01 DA038632, Xu), grant R01-DA2690, and grant R01-AA11330.

The following competing interests for Dr. John H Krystal: 1) **Consultant:** Note: - The Individual Consultant Agreements listed below are less than \$10,000 per year: AstraZeneca Pharmaceuticals; Biogen, Idec, MA; Biomedisyn Corporation; Bionomics, Limited (Australia); Concert Pharmaceuticals, Inc.; Heptares Therapeutics, Limited (UK); Janssen Research & Development; L.E.K. Consulting; Otsuka America Pharmaceutical, Inc.; Spring Care, Inc.; Sunovion Pharmaceuticals, Inc.; Takeda Industries; Taisho Pharmaceutical Co., Ltd; Scientific Advisory Board; Bioasis Technologies, Inc.; Biohaven Pharmaceuticals; Blackthorn Therapeutics, Inc.; Broad Institute of MIT and Harvard; Cadent Therapeutics; Lohocla Research Corporation; Pfizer Pharmaceuticals; Stanley Center for Psychiatric Research at the Broad Institute; 2) **Stock:** ArRETT Neuroscience, Inc.; Blackthorn Therapeutics, Inc.; Biohaven Pharmaceuticals Medical Sciences; Spring Care, Inc. Stock Options: Biohaven Pharmaceuticals Medical Sciences; 3) Income Greater than \$10,000: Editorial Board

Editor - Biological Psychiatry; Patents and Inventions: Seibyl JP, Krystal JH, Charney DS. Dopamine and noradrenergic reuptake inhibitors in treatment of schizophrenia. US Patent #:5,447,948. September 5, 1995; Vladimir, Coric, Krystal, John H, Sanacora, Gerard - Glutamate Modulating Agents in the Treatment of Mental Disorders US Patent No. 8,778,979 B2 Patent Issue Date: July 15, 2014. US Patent Application No. 15/695,164:

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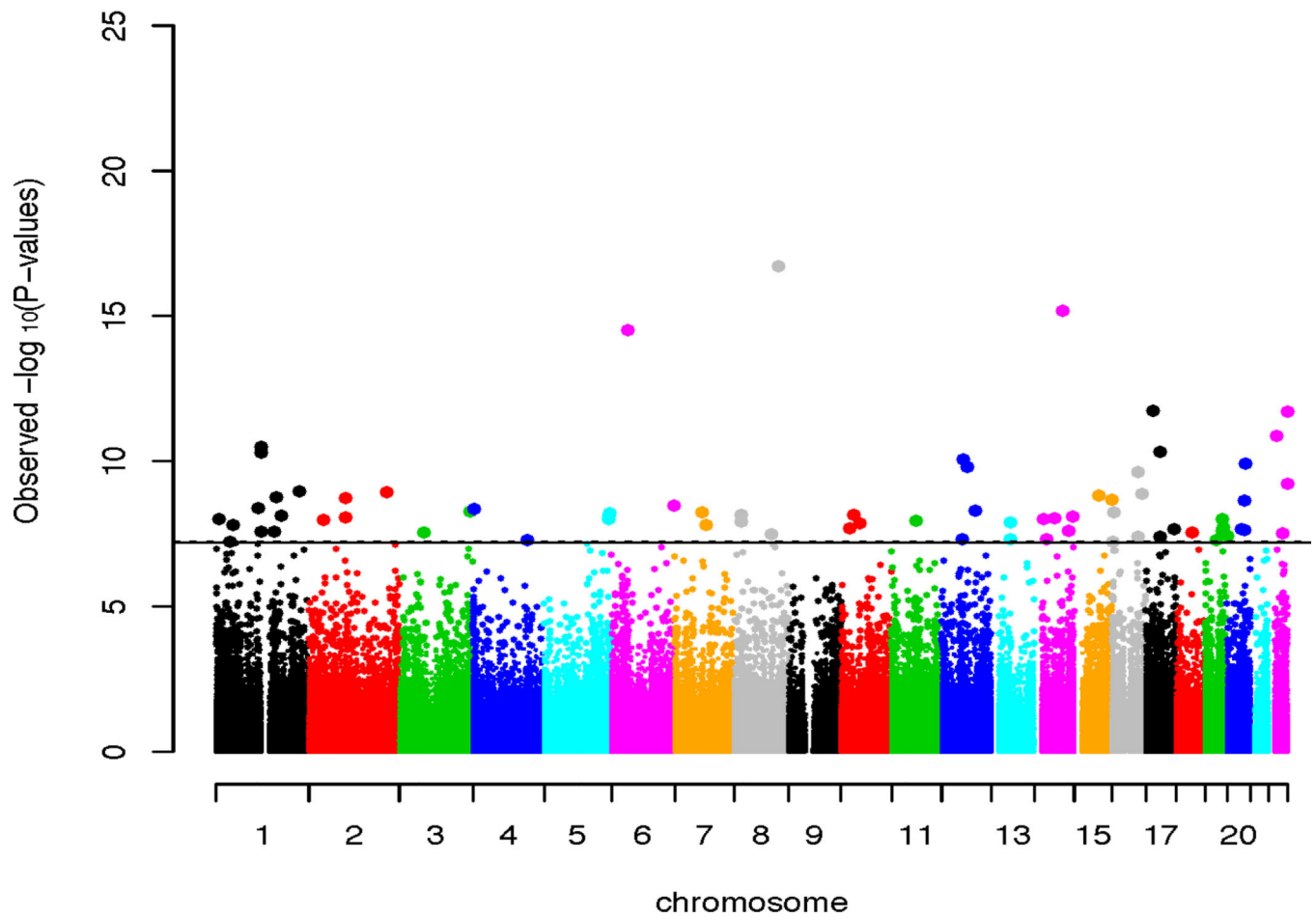


Figure 1. Manhattan plot of epigenome-wide association analysis with 756,573 CpG sites for alcohol consumption. X-axis shows chromosome locations of CpG sites. Y-axis shows $-\log p$ value for each CpG site. The line indicates threshold of epigenome-wide significance equivalent to Bonferroni correction ($p_{\text{nominal}} < 6E-08$). The study was conducted for salivary DNA in 1,135 male veterans with European ancestry.

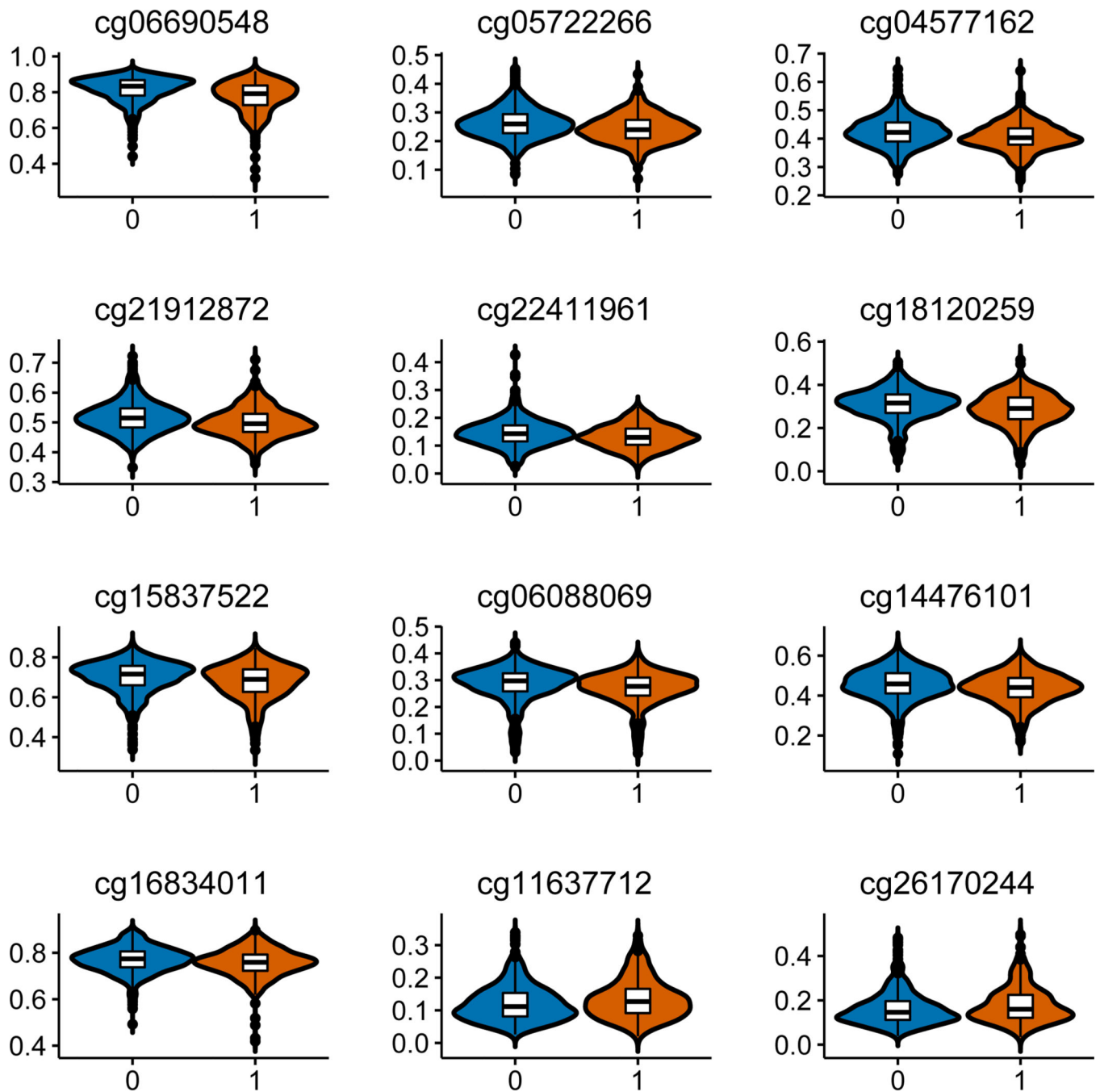


Figure 2. Significant differential DNA methylation on 12 CpG sites between hazardous alcohol consumption and non-hazardous alcohol consumption. X-axis indicates hazardous alcohol consumption =1 and non-hazardous consumption =0; Y-axis indicates average beta-value of DNA methylation for each group. T-test was used to compare beta value between two groups ($p < 0.0007$).

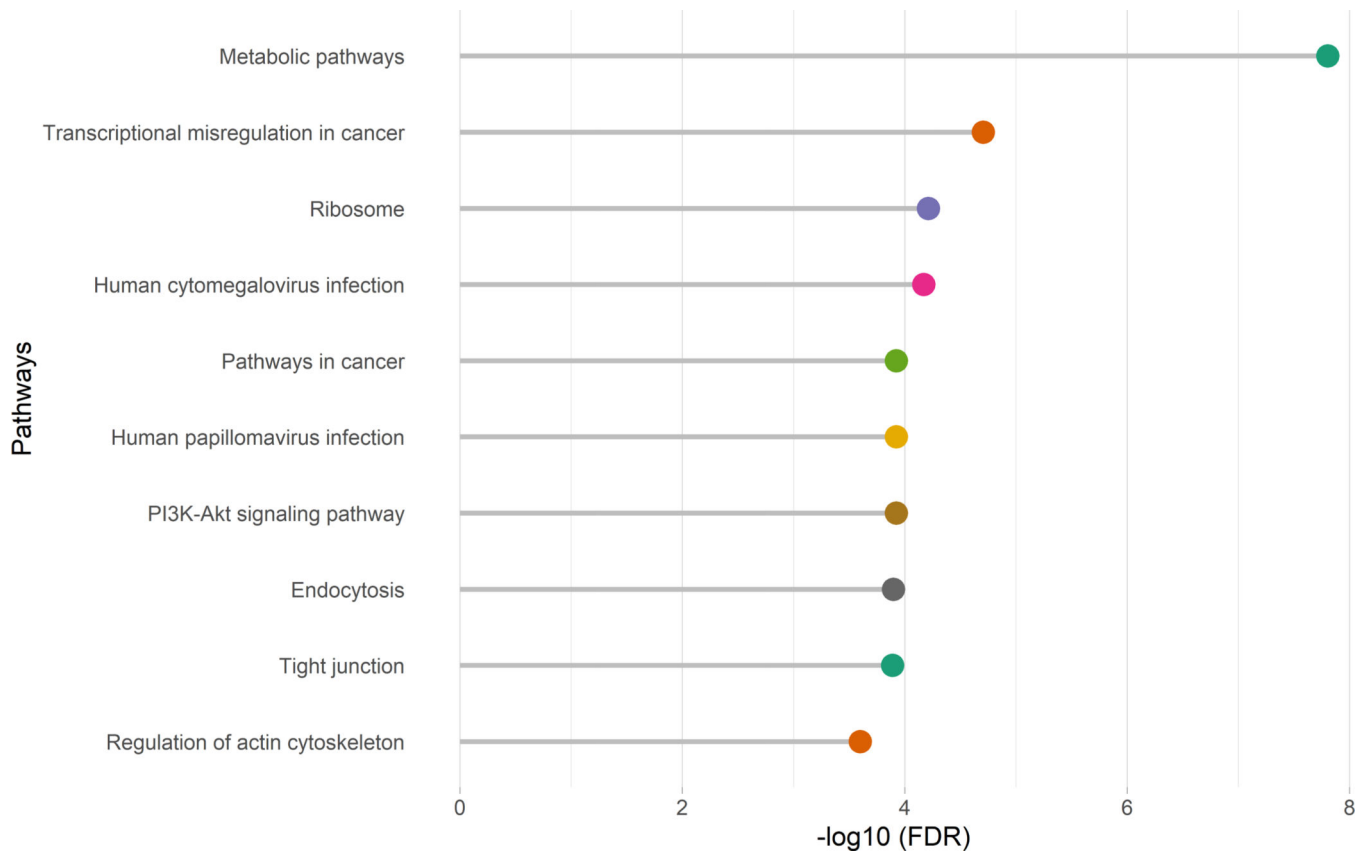


Figure 3. Top 10 significant pathways in the KEGG database for alcohol consumption. CpG sites with $p \leq 5E-04$ in epigenome-wide association analysis were selected for gene set enrichment analysis, which was adjusted with gene length by using missMethyl program in BioConductor. Significant level was set at false discovery rate <0.05 .

Table 1.

Demographic and clinical characteristics between non-hazardous and hazardous alcohol consumption

	None-hazardous Alcohol consumption (N = 807)	Hazardous alcohol consumption (N =328)	p-value
Age (mean ± sd)	64.70 ± 11.96	63.30 ± 12.64	8.55E-02
Sex (male, %)	100	100	1.0
AUDIT_C (mean ± sd)	1.12 ± 1.08	5.33 ± 1.94	3.10E-133
Alcohol use disorder (%)	42.5	62.5	1.49E-09
Current smoking (%)	9.91	17.38	6.77E-04
Nicotine dependences (%)	20.57	21.95	6.62E-01
Drug use disorder (%)	11.28	19.21	5.79E-04
BMI (mean ± sd)	29.15 ± 5.28	27.49 ± 4.47	1.09E-07

Continuous variables: two groups t-test

Binomial variables: two proportions z-Test

Significance: P-value < 5.00E-02

AUDIT_C: Alcohol Use Disorder Identification Test; BMI: Body Mass Index

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Table 2.

Significant CpG sites associated with alcohol consumption in 1,135 male European American veterans with and without adjusted for body mass index

probe	CHR	Position	Gene	GROUP	CPG_ISLAND	t	p	t_adjBMI	p_adjBMI
cg06690548	4	139162808	<i>SLC7A11</i>	Body		-11.35	2.66E-28	-11.70	7.43E-30
cg15837522	8	117892654				-8.64	1.92E-17	-8.96	1.42E-18
cg06088069	14	75895604	<i>JDP2</i>	5UTR	S_Shore	-8.20	6.5E-16	-8.29	3.41E-16
cg18120259	6	43894639	<i>LOC100132354</i>	Body		-8.00	3.05E-15	-8.87	3E-18
cg15696816	17	17713886	<i>RAI1</i>	3UTR	Island	7.13	1.87E-12	7.30	5.34E-13
cg12944146	22	50725609	<i>PLXNB2</i>	Body	Island	7.12	1.97E-12	7.11	2.03E-12
cg16834011	22	19931790	<i>COMT</i>	5UTR	S_Shelf	-6.84	1.33E-11	-6.88	9.98E-12
cg14476101	1	120255992	<i>PHGDH</i>	Body	S_Shore	-6.71	3.17E-11	-7.17	1.38E-12
cg11637712	17	36665844	<i>ARHGAP23</i>	Body	N_Shore	6.65	4.63E-11	6.74	2.62E-11
cg16246545	1	120255941	<i>PHGDH</i>	Body	S_Shore	-6.64	4.95E-11	-7.13	1.88E-12
cg08228578	12	57624193	<i>SHMT2</i>	Body	S_Shore	-6.55	8.91E-11	-6.99	4.88E-12
cg10589813	20	48809978			S_Shore	-6.50	1.25E-10	-6.68	3.74E-11
cg21912872	12	68055270	<i>DYRK2</i>	3UTR		-6.45	1.62E-10	-6.03	2.3E-09
cg06469895	16	69418206	<i>TERF2</i>	Body	N_Shore	-6.39	2.39E-10	-5.97	3.3E-09
cg23041250	22	50725605	<i>PLXNB2</i>	Body	Island	6.25	6.02E-10	6.16	1.02E-09
cg05318849	1	223351252				6.15	1.1E-09	6.18	8.87E-10
cg10440877	2	208378475				-6.13	1.2E-09	-5.71	1.41E-08
cg19138349	16	80040260				6.12	1.33E-09	5.59	2.86E-08
cg26170244	15	65176353				6.09	1.55E-09	5.67	1.85E-08
cg16702725	1	161227516	<i>PCP4L1</i>	TSS1500	N_Shore	6.07	1.76E-09	6.16	9.99E-10
cg05722266	2	96991791	<i>ITPRIPL1</i>	TSS200	S_Shore	-6.06	1.9E-09	-5.96	3.31E-09
cg22411961	15	99318650	<i>IGF1R</i>	Body		-6.04	2.13E-09	-5.55	3.52E-08
cg01764256	20	45985832	<i>ZMYND8</i>	TSS200		-6.03	2.21E-09	-5.92	4.28E-09
cg03849851	6	166980629	<i>RPS6KA2</i>	Body		5.96	3.32E-09	5.59	2.83E-08
cg00035969	1	112159097			N_Shelf	5.93	4.1E-09	5.79	8.99E-09
cg01903185	4	1808586	<i>FGFR3</i>	Body	S_Shore	5.92	4.37E-09	5.83	7.34E-09
cg00508575	12	90050967	<i>ATP2B1</i>	TSS1500		5.89	5.02E-09	5.74	1.24E-08
cg09326269	3	189429439	<i>TP63</i>	Body		5.88	5.29E-09	5.57	3.17E-08
cg04577162 *	7	73667397	<i>RFC2</i>	Body	N_Shore	-5.87	5.79E-09	-5.18	2.67E-07
cg04725426	16	2131752	<i>TSC2</i>	Body	N_Shelf	5.87	5.86E-09	5.90	4.75E-09
cg16968128	5	176810555	<i>SLC34A1</i>	TSS1500		5.85	6.31E-09	5.74	1.22E-08
cg21998542	10	33605101	<i>NRP1</i>	Body		-5.84	6.93E-09	-5.91	4.64E-09
cg07020366	8	19246614	<i>SH2D4A</i>	Body		5.83	7.19E-09	5.37	9.71E-08
cg06644515	1	173834831	<i>SNORD47/ SNORD80</i>	TSS1500	N_Shelf	-5.82	7.5E-09	-5.44	6.49E-08
cg17962756	5	172769199				-5.82	7.91E-09	-5.65	2.1E-08

probe	CHR	Position	Gene	GROUP	CPG_ISLAND	t	p	t_adjBMI	p_adjBMI
cg21050667	14	103624533				5.82	7.91E-09	5.59	2.8E-08
cg06739059	2	96991826	<i>ITPR1PL1</i>	5UTR	S_Shore	-5.80	8.65E-09	-5.68	1.75E-08
cg13409544	14	53159218	<i>ERO1A</i>	Body	N_Shelf	5.79	9.34E-09	5.63	2.32E-08
cg26250989	1	6333502	<i>ACOT7</i>	Body		5.78	9.55E-09	5.57	3.28E-08
cg13526915	14	24164078				-5.78	9.75E-09	-5.85	6.4E-09
cg07463740	5	172737556				-5.77	1.01E-08	-5.55	3.63E-08
cg10052860	19	45189438				5.77	1.01E-08	5.37	9.76E-08
cg05190930	2	38701258	<i>LOC101929596</i>	Body		5.77	1.04E-08	5.57	3.22E-08
cg13823144	11	66475721	<i>SPTBN2</i>	Body		5.76	1.1E-08	5.68	1.71E-08
cg13825056	8	17186545	<i>MTMR7</i>	Body		-5.74	1.22E-08	-5.53	4E-08
cg11408657	13	49147568				5.74	1.25E-08	5.76	1.09E-08
cg27141751	10	50231850	<i>C10orf72</i>	Body		5.72	1.39E-08	5.52	4.27E-08
cg03533141	1	45243518	<i>SNORD38A</i>	TSS1500	S_Shore	-5.70	1.54E-08	-5.20	2.43E-07
cg07446048	7	82353289				-5.69	1.58E-08	-5.20	2.41E-07
cg07626482	19	47289503	<i>SLC1A5</i>	TSS1500	N_Shore	-5.66	1.91E-08	-6.02	2.34E-09
cg00375132	10	22768370			S_Shore	-5.65	2.02E-08	-5.45	6.22E-08
cg05476232	17	74361501				5.64	2.12E-08	5.43	6.8E-08
cg13564967	20	36771794	<i>TGM2;TGM2</i>	Body		5.64	2.17E-08	5.39	8.76E-08
cg01385679 *	20	45033155	<i>ELMO2</i>	5UTR	N_Shore	-5.63	2.27E-08	-5.23	2.08E-07
cg16940062	14	91763798	<i>CCDC88C</i>	Body		5.61	2.5E-08	5.76	1.11E-08
cg09109179	19	45268492				5.61	2.53E-08	5.49	5.06E-08
cg26457483	1	120256112	<i>PHGDH</i>	Body	S_Shore	-5.60	2.73E-08	-5.99	2.79E-09
cg19458269 *	1	155949421	<i>ARHGEF2</i>	TSS1500	S_Shore	-5.60	2.74E-08	-5.31	1.32E-07
cg24644830 *	3	66533732	<i>LRIG1</i>	Body		-5.59	2.84E-08	-5.043	5.36E-07
cg01589532 *	22	38696599	<i>CSNK1E</i>	Body		5.57	3.13E-08	5.507	4.55E-08
cg06838966	8	98852306	<i>LAPT4B</i>	Body		5.57	3.28E-08	5.51	4.55E-08
cg11701312	19	58897497	<i>RPS5</i>	TSS1500	N_Shore	-5.54	3.75E-08	-5.11	3.86E-07
cg13545705 *	16	67312573	<i>PLEKHG4</i>	TSS1500	N_Shore	-5.53	4E-08	-4.11	4.29E-05
cg12192847 *	17	36641717	<i>ARHGAP23</i>	Body		5.53	4.04E-08	5.53	3.94E-08
cg12772028	14	33080373	<i>AKAP6</i>	Body		5.50	4.7E-08	5.44	6.6E-08
cg02583484 *	12	54677008	<i>HNRNPA1</i>	Body	S_Shelf	-5.50	4.83E-08	-4.10	4.46E-05
cg25989526 *	13	49147573				5.49	4.87E-08	5.26	1.70E-07
cg08151621 *	19	28995456	<i>LOC100420587</i>	Body		-5.48	5.25E-08	-5.22	2.16E-07
cg04425005	4	146783253	<i>ZNF827</i>	Body		-5.48	5.28E-08	-5.52	4.32E-08
cg10254445	1	37197260				-5.46	5.75E-08	-5.40	7.98E-08

Note:

* CpG sites with suggestive epigenom-wide significance after adjusted for body mass index (BMI)