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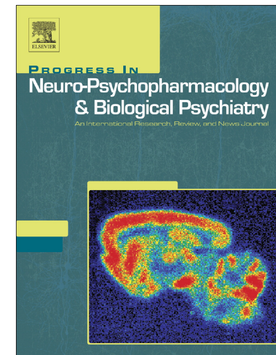

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Genetic variation and epigenetic modification of the prodynorphin gene in peripheral blood cells in alcoholism

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ABSTRACT

Dynorphins are critically involved in the development, maintenance and relapse of alcoholism. Alcohol-induced changes in the prodynorphin gene expression may be influenced by both gene polymorphisms and epigenetic modifications. The present study of human alcoholics aims to evaluate DNA methylation patterns in the prodynorphin gene (*PDYN*) promoter and to identify single nucleotide polymorphisms (SNPs) associated with alcohol dependence and with altered DNA methylation. Genomic DNA was isolated from peripheral blood cells of alcoholics and healthy controls, and DNA methylation was studied in the *PDYN* promoter by bisulfite pyrosequencing. In alcoholics, DNA methylation increased in three of the seven CpG sites investigated, as well as in the average of the seven CpG sites. Data stratification showed lower increase in DNA methylation levels in individuals reporting craving and with higher levels of alcohol consumption. Association with alcoholism was observed for rs2235751 and the presence of the minor allele G was associated with reduced DNA methylation at *PDYN* promoter in females and younger subjects. Genetic and epigenetic factors within *PDYN* are related to risk for alcoholism, providing further evidence of its involvement on ethanol effects. These results might be of relevance for developing new biomarkers to predict disease trajectories and therapeutic outcome.

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INTRODUCTION

Alcohol abuse causes about 1.8 million deaths a year (3.2 % of all deaths worldwide), and alcohol dependence with associated health problems is the third most common cause of death in developed countries (Rehm et al., 2004). Continued excessive alcohol consumption can lead to the development of alcohol dependence, characterized by physiological withdrawal when alcohol consumption is substantially reduced. Unpleasant withdrawal effects contribute to relapse, estimated to occur in 50 – 90 % of abstinence attempts, and are clearly a major impediment to treatment efforts (Loosen PT et al., 1990; Powell BJ et al., 1998; Finney JW et al., 1999; Ciraulo D et al., 2003; Moos RH & Moos BH, 2006; Charney et al., 2010). Increasing evidence supports a link between alcohol consumption and the endogenous opioid system, which is proven to be important when considering positive reinforcing effects following acute alcohol consumption and the downstream neuroadaptations following chronic alcohol exposure (Walker et al., 2012). In animal models, acute alcohol stimulates endogenous opioid peptide release (Lam et al., 2008), whereas a central opioid deficiency has been observed during heavy alcohol consumption, which might further promote alcohol intake through negative reinforcement. β-endorphin and enkephalin, endogenous ligands of the mu-opioid receptor (MOP) and delta-opioid receptor (DOP), respectively, have been associated with the euphoric/rewarding effects of alcohol consumption (Nealey et al., 2011).

In contrast, dynorphins (DYNs), endogenous ligands of the kappa-opioid receptor (KOP) (Chavkin et al., 1982) induce dysphoric/anhedonic effects in humans and aversive behaviors in animals (Nealey et al., 2011), contributing to excessive alcohol seeking, similar to that observed in alcohol dependence (Walker and Koob, 2008). It has also been suggested that the DYNs/KOP system may be a key mediator in the stress-related effects of alcohol (Walker et al., 2012). Alcohol-induced changes in the prodynorphin gene (*PDYN*) expression have been linked to neuroplastic adaptations critical for addiction (Bazov et al., 2013; Butelman et a., 2012; D'Addario et al., 2008 and 2013; Shippenberg et al., 2007; Taqi et al., 2011a; Walker and Koob, 2008; Wee and Koob, 2010), contributing to the impairment of cognitive control over alcohol drinking behavior (Shippenberg et

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al., 2007; Taqi et al., 2011a and 2011b). Changes in the *PDYN* gene expression may be influenced by gene polymorphisms and epigenetic mechanisms. It is, in fact, known that development of alcoholism is influenced by both genetic and environmental factors (Gelernter and Kranzler, 2009; Kimura and Higuchi, 2011), and that gene-environment interactions can be mediated by epigenetic regulation of gene expression. The *PDYN* gene remains a strong candidate for alcohol dependence-related phenotypes in humans, even though genetic studies on the link between *PDYN* variation and alcohol dependence have demonstrated both association (Karpyak et al., 2013;Williams et al., 2007; Xuei et al., 2006) as well as lack of association (Bierut et al., 2010; Edenberg et al., 2010; Treutlein et al., 2009). However, the effects of previously detected genetic risk factors for psychiatric disorders, including alcohol dependence, remain small.

We explore here the hypothesis that the influence of alcohol consumption on human physiology might be due to a combination of genetic and environmental factors, which are mediated by epigenetic mechanisms. DNA methylation is one of the most studied epigenetic modifications, associated with reduced gene expression, and is known to be involved in numerous biological processes (Bird, 2002). Genetic variations have been shown to influence the inter-individual variation in DNA methylation (Zhang et al., 2010). However, the impact of genetic variations on the DNA methylation pattern of most genes is not fully understood. The aim of our study is to identify the genetic and epigenetic regulation of *PDYN* in alcoholism by: (A) analysis of DNA methylation of the proximal promoter region of *PDYN* in peripheral blood cells from alcoholics and healthy controls; and (B) detection of single nucleotide polymorphisms (SNPs) in the *PDYN* gene associated with alcohol dependence and/or DNA methylation changes. The availability of a well-defined cohort of alcoholics allows us to use data stratification based on different characteristics of the study sample and thus to possibly assess disease development and trajectories.

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METHODS

Study subjects and data collection

This study was approved by the Regional Ethics Review Board in Stockholm, Sweden, for the alcoholic and control samples (Number: Dnr:01-392). All alcoholic subjects provided informed consent and permission to use their information for future studies of alcohol dependence and related phenotypes. Samples from 744 affected individuals (521 males of mean age 49.4 ± 10.7 years; 223 females of mean age 48.6 ± 10.4 years) and 1079 control subjects (301 males and 778 females of mean age 52 ± 12 years) of Scandinavian ancestry were included in the association study. Alcoholic patients were enrolled within the Swedish 'Alcoholism in Siblings' study. Alcohol dependence was defined according to DSM-IV criteria after a structural psychiatric interview conducted in accordance with the Schedules for Clinical Assessment in Neuropsychiatry (Wing et al., 1990). Subjects who additionally met DSM-IV criteria for dependence on an illicit drug or had recently (within one year) suffered any major psychiatric disorder were excluded. A history of psychiatric disorders, such as a depressive episode, did not lead to exclusion. The group of healthy controls consisted of individuals enrolled in the 'Epidemiological Investigation of Rheumatoid Arthritis (EIRA)' study (Plenge et al., 2007). These control individuals for the genetic association study were Swedish Caucasians from the general population between the ages of 18 and 70 years and were included in the study during the period 1996-2004. Data for alcohol consumption was available for 955 individuals (88.5%); 113 (11.8%) declared that they never consumed alcohol; for the remaining individuals, alcohol consumption was skewed to low values with a median of 48 g per week, IQR 50. Demographic and clinical characteristics of the alcoholic patient cohort are shown in Table 1.

SNP selection and genotyping

Genomic DNA was isolated as previously described (Geijer et al., 1994). Locations of the selected *PDYN* SNPs examined are shown in Figure 1a. Candidate SNPs for genotyping were chosen to replicate previously reported associations with alcohol, opiate and/or cocaine dependence (Xuei et al., 2006; Yuferov et al., 2009). Non-alcoholic controls were genotyped as part of EIRA study

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(Plenge et al., 2007). TaqMan Genotyping Assays were applied for genotyping of SNPs (Life Technologies, Carlsbad, CA) for cases and controls. The end point fluorescence readings were performed using an ABI Prism 7900 System.

Analysis of DNA methylation by bisulfite pyrosequencing

A subset of 151 alcoholics and 88 healthy individuals was selected for the methylation studies to include individuals for statistical analysis that were representative of the total study population with regard to sex, age distribution, genotype and, for alcoholics, the phenotypes distribution. Methylation status of the *PDYN* promoter region was determined using pyrosequencing of bisulfite-converted genomic DNA isolated from blood cells. After DNA extraction, 0.5 µg of DNA from each sample was treated with bisulfite, using a DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulfite-treated DNA was amplified by the PyroMark PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. *PDYN* primer sequences (Forward: GGAAGAGAGTAGGAAGATGATAG; biotin-Reverse: TTCTACCAAAACTAATATACCTAACACC; Sequencing: AGAGAGTAGGAAGATGATAGT) were provided by Qiagen. The sequence was from the forward strand and the assay was designed to target a region within the CpG island located upstream of the *PDYN* exon 1 (Figure 1b) previously identified by Yuferov et al., 2011. PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles of 94°C for 30s, 56°C for 30s, 72°C for 30s, and, finally, 72°C for 10 min. PCR products were verified by agarose electrophoresis. Pyrosequencing methylation analysis was conducted using the PyroMark Q24 (Qiagen). The level of methylation was analyzed using PyroMark Q24 Software (Qiagen), which calculates the methylation percentage (mC/(mC+C)) for each CpG site, allowing quantitative comparisons (mC is methylated cytosine, C is unmethylated cytosine). Intra-assay variability was assessed using the coefficient of variation between investigated CpG sites (Table S1). Human fully methylated and unmethylated DNA (Zymo Research (Irvine, CA, USA) were bisulfite-converted and mixed to obtain the following ratios of methylation: 0%, 25%, 50%, 75%, 100%, and standard curves were used to confirm linearity of the assay. R values for each CpG site

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are also reported in Table S1.

Statistical Analysis

Statistical calculations were performed using the JMP software package, version 12.0.1 (SAS Institute Inc., NC, USA). First, DNA methylation at each CpG site was analyzed using the Mann Whitney test. Second, data were adjusted in a standard least square model for age and sex. Adjustment for smoking was performed for selected analyses. Finally, Bonferroni correction for eight comparisons was used for the individual parameters of this model (alcoholics or control group) and this number will be referred to as corrected *P*-value in the text. The Pearson chi-square test was applied to assess the significance of the genotyping and allelic association of each variant.

RESULTS

DNA methylation of PDYN promoter region

Bisulfite pyrosequencing was performed on a portion of the *PDYN* CpG island (Figure 1). We were able to obtain valid data for 7 CpG sites, out of 9 present, in this region and found that the average methylation across these sites increased significantly in DNA from peripheral blood cells of alcoholics relative to controls (controls: 83.79 ± 0.70; alcoholics: 85.85 ± 0.25; corrected *P* = 0.0078) (Figure 2a). Analyzed individually, a significant increase in DNA methylation was observed in DNA from peripheral blood cells of the alcoholics compared to controls at CpG sites number 3 (controls: 92.54 ± 0.89; alcoholics: 95.05 ± 0.23; *P* = 0.0061) and number 5 (controls: 92.14 ± 1.14, alcoholics: 95.01 ± 0.31; *P* = 0.0279) (Figure 2b).

Considering just the alcoholic population, DNA methylation differences were not observed after data stratification based on gender, age, presence of withdrawal, relief drinking (meaning that an aversive stimulus is terminated by drinking alcohol) and tolerance (Figure S1). Instead, in subjects reporting craving as well as in those with higher frequency of drinking, we observed a reduction in the percentage of DNA methylation at CpG 7 (none/low alcohol exposure: 76.71 ± 0.58, high alcohol exposure: 72.058 ± 0.75, *P* = 0.0316; non reporting craving: 75.21 ± 0.67, reporting

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craving: 71.75 ± 0.91, P = 0.048), and in the average percentage for all 7 CpG sites (none/low alcohol exposure: 86.78 ± 0.37, high alcohol exposure: 85.32 ± 0.34, P = 0.0428; non reporting craving: 86.42 ± 0.31, reporting craving: 84.92 ± 0.44: P = 0.0051) (Figure 3 a; b).

Higher levels in DNA methylation were observed in alcoholic males (CpG 4: controls: 77.76 ± 2.49, alcoholics: 84.26 ± 0.73, P = 0.0359; CpG 7: controls: 63.46 ± 2.96, alcoholics: 73.56 ± 0.83, P = 0.018; average of 7 CpG sites: controls: 81.86 ± 1.39, alcoholics: 85.95 ± 0.33, P = 0.0084) and older alcoholic subjects (older then 40 years) (CpG 5: controls: 92.27 ± 1.26, alcoholics: 95.39 ± 0.27, P = 0.0241; CpG 7: controls: 67.30 ± 1.83, alcoholics: 73.03 ± 0.82, P = 0.0109; average of 7 CpG sites: controls: 83.33 ± 0.82, alcoholics: 85.70 ± 0.32, P = 0.0044) when compared with respective matching healthy controls (Figure 4b and 4d), whereas younger alcoholics (under 40 years old) have significant lower levels of the epigenetic mark with respect to healthy subjects within the same range of age (CpG 4: controls: 87.58 ± 1.90, alcoholics: 81.88 ± 1.23, P = 0.05; CpG 7: controls: 76.89 ± 1.50, alcoholics: 71.47 ± 1.35, P = 0.05) (Figure 4c). Moreover, higher levels of the epigenetic mark were also observed at CpG 3 in alcoholic females (controls: 93.13 ± 0.69, alcoholics: 95.38 ± 0.42, P = 0.0054).

We also observed a significant increase in DNA methylation at CpG site number 4 in alcoholic males with higher levels of alcohol exposure (females: 80.22 ± 1.49, males: 84.18 ± 0.62, P = 0.0363) and reporting marked tolerance (females: 80.08 ± 1.83, males: 84.61 ± 0.67, P = 0.0427), when compared to alcoholic females (Figure S2).

Association of PDYN SNPs with alcoholism

We tested genotypes for 10 SNPs across the *PDYN* gene, from 4 kb upstream of the transcription initiation site to 3 kb downstream of the 3' end, in 744 alcoholics and 1079 controls (Figure 1a). Results of analyses of association between alcohol dependence and *PDYN* SNPs are presented in Table 2. We found that carriers of the minor allele C of rs2281285 SNP demonstrated an association with alcoholism in the female population (P = 0.035), as did subjects carrying the minor

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allele G of rs2235751 ($P = 0.036$) (Table 1).

Genetic association with DNA methylation

To determine the extent of genetic regulation of the level of methylation we compared DNA samples from peripheral blood of alcoholics and controls with different genotypes. We found that the presence of the minor allele G of SNP rs2235751 was associated with a significant reduction in DNA methylation at CpG 5 (controls: 95.98 ± 1.04, alcoholics: 92.34 ± 1.03; P = 0.0403) in alcoholics, whereas major allele carriers show higher levels of DNA methylation at CpG 3 (AA: controls: 91.65 ± 1.83, alcoholics: 94.89 ± 0.32; P = 0.0484; AG: controls: 93.10 ± 1.08, alcoholics: 95.67 ± 0.34; P = 0.0012) and at CpG 5 (AG: controls: 93.23 ± 1.07, alcoholics: 95.80 ± 0.32; P = 0.015) (Figure 5). Moreover, within alcoholic subjects, when data were stratified for gender and age, the presence of the minor allele for SNP rs2235751 was found in association with lower DNA methylation for CpG number 4 (males: 83.14 ± 1.16, females: 77.80 ± 2.13; P = 0.02), CpG 5 (males: 93.45 ± 0.66, females: 90.47 ± 2.21; P = 0.016), CpG 6 (males: 82.18 ± 1.18, females: 77.24 ± 3.14; P = 0.0075), CpG 7 (males: 73.06 ± 1.62, females: 66.11 ± 2.62; P = 0.015) and in the average of the 7 CpG sites in females (males: 85.01 ± 0.71, females: 81.66 ± 2.03; P = 0.013) (Figure 6a), and for CpG number 5 (under 40 y: 90.59 ± 1.69, over 40 y: 94.51 ± 0.68; P = 0.0048) and in the average of the 7 CpG sites in younger subjects (under 40 y: 84.00 ± 1.08, over 40 y: 85.29 ± 0.91; P = 0.025) (Figure 6b). Figure S3 reports in more details these changes in DNA methylation for the different genotypes based on gender and age. A significant correlation between age and DNA methylation levels at the average of the seven CpG sites in alcoholics was also observed (P = 0.0050) but not in control subjects (Figure S4).

It should be mentioned that after Bonferroni correction for multiple testing, the association remains for all the stratification data for the average of the seven CpG sites, whereas it does not survive for the single CpG sites.

DISCUSSION

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There is strong evidence that methylation of DNA, together with specific modifications of histones, has a major influence on chromatin structure and regulates gene expression (Cedar and Bergman, 2009). The major finding of our study is that DNA methylation is increased in peripheral blood cells of alcoholics when compared to healthy controls in 2 out of the 7 CpG sites under evaluation in the CpG island within the proximal *PDYN* gene promoter. We also see a trend towards correlation between DNA methylation and the genetic variant in the *PDYN* promoter region in alcoholics. This finding, which is in line with brain tissue data on a different genetic variation (Taqi et al., 2011), provides support for suggesting further investigation of blood cell methylation profile as a proxy for epigenetic changes in the brain.

The possible role of the *PDYN* gene promoter CpG island in gene expression regulation has been previously investigated, and was shown to cause up-regulation (Carrion et al., 1998) or down-regulation (Rouault et al., 2011) of basal and forskolin-induced *PDYN* promoter activity – effects that are likely to be cell type specific. The data reported here are in agreement with a previous study showing an increase in DNA methylation of a CpG-SNP, at the level of *PDYN* 3'-UTR, in the cortex of human alcohol-dependent subjects (Taqi et al., 2011). This is also of relevance when taking into account the similarity of CpG methylation patterns of *PDYN* gene in brain tissues and peripheral blood cells reported by Yuferov and colleagues (Yuferov et al., 2011).

While no differences in DNA methylation levels were observed with respect to withdrawal history or alcohol tolerance, a reduction of these levels was detected in DNA from subjects reporting craving and in those with higher levels of alcohol consumption. These observations further stress the positive correlation between *PDYN* gene expression and the desire to drink. Dynorphins are known to produce aversive, dysphoric-like effects (Shippenberg et al., 2007). The lower level of DNA methylation at the *PDYN* gene promoter in these alcoholics probably leads to up-regulation of *PDYN* gene expression in individuals experiencing the negative reinforcing effects of alcohol and thus these individuals have a level of DNA methylation close to the level of the control group. Changes in the epigenetic mark seems to be dependent on subjects age and sex, in fact alcoholics

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over 40 years old (Figure 4d and Figure S4) and males (Figure 4b) have higher levels of DNA methylation when compared age matching control subjects.

We did not observe gender differences in *PDYN* DNA methylation levels, however females reporting high alcohol exposures and marked tolerance show lower levels of DNA methylation when compared to males.

Consistently, down-regulation of *PDYN* mRNA, possibly associated with the increase in DNA methylation, has been also reported recently in the putamen and caudate nucleus in human alcoholics (Sarkisyan et al., 2015). The same authors previously reported up-regulation of *PDYN* in the cortex and hippocampus of alcoholics (Bazov et al., 2013) and this difference has been related to the specific roles of the different brain areas. Thus, the reduction in *PDYN* gene expression specifically in the striatum, and in blood as seen in the present study, might relate to the formation of a compulsive habit evoked by the chronic alcohol consumption, or be an inherent feature of alcoholics, whereas the increase in mRNA in the cognitive areas might be due, as suggested, to the impairment of cognitive control of addictive behavior (Sarkisyan et al., 2015). High levels of striatal dynorphins have been also suggested to play a protective function by preventing both the development and maintenance of substance dependence (Yuferov et al., 2011). Thus, we might speculate that the PDYN epigenetic regulation in blood, as reported here, might be due to the actual occurrence of alcohol dependence in the study sample. It should be also mentioned that the same genomic region we here studied has been previously found to induce up-regulation or down-regulation of PDYN expression (Carrion AM et al., 1998; Rouault M et al., 2012), thus the changes observed do not necessarily reflect a reduction in gene expression.

To speculate whether the elevated DNA methylation is a consequence of chronic alcohol consumption or an inherent property of alcoholics, we explored the possible interaction between genetic and epigenetic factors. A growing amount of evidence points to the importance of both environmental and genetic factors in influencing DNA methylation, and a hypothetical model for their interaction in the predisposition to, and development of, alcoholism has been suggested

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(Starkman et al., 2012).

Association data for alcohol-related phenotypes in our study demonstrated, at best, a moderate effect from genetic variations in *PDYN*. DNA methylation can be influenced by cis-acting DNA sequence variations located on the same chromosome (Docherty et al., 2012; Milani et al., 2009; Mill et al., 2008; Zhang et al., 2010). The association of epigenetic changes with DNA-sequence variations might also explain why genetic association studies in complex diseases are frequently inconsistent. It is thus clear that new models have to be developed to integrate genetic variants and DNA methylation. In fact, SNPs and CpG sites might jointly contribute to target gene expression and considering both genetic variants and DNA methylation might be of relevance in the attempt to improve the understanding of alcoholism in the context of our work, as well as, in a wider context, of other diseases. Among the ten SNPs, chosen on the basis of previous linkage or association studies, only the minor allele of rs2235751 variant showed an association with propensity to use alcohol in the overall population, whereas rs2281285 variant was marginally associated with alcoholism just in the female population, as already reported by Xuei *et al.*, 2006 and Karpyak *et al.*, 2013, respectively. SNP rs2281285 is located in intron B of *PDYN*, 769 bp downstream of exon 2/intron B junction (Nikoshkov et al., 2005) where the allelic variability may contribute to differential regulation of these two transcripts (Karpyak et al., 2013). A trend towards association of the rs2281285 minor allele with increased risk for alcohol dependence was recently reported, but only in males and not in females (Winham et al., 2015). However, the authors also reported in female alcoholics an association between the rs2281285 minor allele and increased negative craving and risk of relapse. Thus, the apparent discrepancies in our findings with these previously reported results on gender-dependent association of the rs2281285 minor allele may be explained by different factors potentially influencing the direction of the association (e.g. presence/absence of co-morbid disorders, the impact of other genetic or epigenetic variations). We did not observe in our study population association with alcoholism for rs1997794 or rs910080, as previously reported (Xuei et al., 2006).

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We further focused our attention on rs2235751, linked to alcoholism in the entire population, to evaluate SNP association with DNA methylation. It is thus possible to hypothesize an impact of genetic control on DNA methylation: in alcoholic subjects carrying the minor allele the level of DNA methylation is lower when compared to healthy subjects as well to alcoholics carrying the major allele. Again, this effect is affected by gender and age. In fact, younger alcoholics and females carrying the minor allele have lower levels of DNA methylation when compared to older subjects and males. This might suggest that a lower DNA methylation in particular in younger and females alcoholics, might mask the possible predisposition effect of the SNP and thus reduce the risk of alcoholism. This observation is of particular relevance in the attempt to explain why SNPs in candidate genes including *PDYN* are not replicated in studies of alcoholism. It would be thus important to consider, together with possible alterations of epigenetic marks, potential population stratification such as gender and age as in the case of this report.

Our data on the hypermethylation of the *PDYN* gene, possibly leading to reduction in its gene expression, are in contradiction with animal studies that report an increase in dynorphin activity following chronic alcohol exposure (Shippenberg et al., 2007; Walker et al., 2011; Wee and Koob, 2010). However, as already proposed (Taqi et al., 2011), these differences might be due to the phase of the addiction cycle, with an upregulation during the development of dependence and a decrease during the phase of maintenance; this latter phase might be the general condition of the alcoholics in this study.

It could be argued that the DNA methylation changes observed are too small to have any functional relevance. However, it is now clear that complex phenotypes can be influenced by multiple factors with small individual effects (Lupski et al., 2011; Wellcome Trust Case Control Consortium, 2007). Thus, as already suggested, subtle differences in DNA methylation levels could be relevant and many small differences in the epigenetic landscape could be responsible for the different phenotypic outcome in the population (Nikoshkov et al., 2005). Another possible concern is the distance between the CpG sites under study and rs2235751. These CpG sites are located ≈ 15,000 bp away

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from the SNP in the intragenic region of the *PDYN* gene upstream to the gene; however, it has been demonstrated that regulation in cis arrangement can occur over even greater distances (Bell et al., 2011). Finally, a possible limitation of the study could be that genotyping was performed separately in alcoholic subjects and controls, which may potentially bias study results. However, this is unlikely since our analysis indicates low levels of genotyping errors.

An important limitation of our study is that whole blood is a heterogeneous population of different cell types, each with a very different DNA methylation profile (Jaffe and Irizarry, 2014). However, it should be noted that these confounding factors are not confined to blood, but rather apply to any tissue that contains a mixture of cell types (Whitney et al., 2003), brain included. Moreover, it should also be considered that extraction of more homogeneous cell populations is often laborious, difficult to standardize and involves cells manipulation and thus may influence the expression profiles (Debey et al., 2004). Thus, to overcome these limitations, novel epigenetic approaches using cell-sorting or laser capture microdissected tissues might be adopted.

This is the first study to jointly investigate combined effects of genetic and epigenetic factors within the genomic region of the *PDYN* gene and the risk for alcoholism. Moreover, consistent with the mirror-site model stating that the methylation status of many sites in the blood mirrors those in the brain (Aberg et al., 2013), we here suggest a new hypothesis to explain the well-established ethanol effects on the regulation of a key element of the endogenous opioid system. There is an urgent need for biomarkers in psychiatric disorders, including alcoholism, and our study suggests that specific genetic variation and/or epigenetic modulation of *PDYN* gene might fulfill this function. The understanding of gene-environment interactions is extremely important in the study of alcoholism and its treatment (epi-treating factors), and the possible use of drugs targeting the *PDYN* gene, taking advantage of the reversible nature of epigenetics, might be promising. Further association studies are needed in the attempt to suggest other targets, and thus possible biomarkers to complement DYN, and also to identify other possible forms of pharmacotherapy for alcoholism.

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Authors contribution

Conceived and designed the experiments: CD MP. Performed the experiments: CD MP KP AG. Analyzed the data: CD CC LP VV. Contributed reagents/materials/analysis tools: CD CC LT.

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Wrote the paper: CD LP LT.

All authors have contributed to and have approved the final manuscript.

LEGEND TO FIGURES

Legend to Figure 1

(a) Schematic representation of the human *PDYN* gene and the 5' upstream region. ATG is the translation start site. Shown are the locations of the CpG island, Exons I-IV and the position of the SNPs genotyped. Coding regions of exons are shown as black, and 5' and 3' untranslated regions (UTR) as white boxes. (b) The sequence of the CpG island (chr 20: 1994648-1994795) that was used for DNA methylation analyses. Bold text indicates the 7 CpG sites (out of 9 present in the island) analyzed to evaluate the methylation status.

Legend to Figure 2

Comparison of the DNA methylation status in the human *PDYN* gene promoter between alcoholics and controls. Average methylation of the 7 CpG sites (a) as scatter plot and (b) bars of individual CpG sites under study. The bars represent the mean of the % of methylation values of individual CpG sites under study ± the S.E.M. Significant differences are indicated (Mann-Whitney test): * $P < 0.05$; ** $P < 0.01$.

Legend to Figure 3

Comparison of the DNA methylation status at human *PDYN* gene promoter in the alcoholic population stratified based on (a) level of alcohol exposure (defined as number of drinking days/month) and (b) reported of craving. The bars represent the mean of the % of methylation values of individual CpG sites under study as well as of the average (AVE) of the 7 CpG sites ± the S.E.M. Significant differences are indicated (Mann-Whitney test): * $P < 0.05$; ** $P < 0.01$.

Legend to Figure 4

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Comparison of the DNA methylation status at human *PDYN* gene promoter in the alcoholic population stratified based on gender (a) and (b) or age (c) and (d). The bars represent the mean of the % of methylation values of individual CpG sites under study as well as of the average (AVE) of the 7 CpG sites ± the S.E.M. Significant differences are indicated (Mann-Whitney test): * $P < 0.05$; ** $P < 0.01$.

Legend to Figure 5

Comparison of the DNA methylation status at the human *PDYN* gene promoter in the controls and alcoholic population stratified by the genotype for rs2235751, where A is the major allele and G is the minor allele. The bars represent the mean of the % of methylation values of individual CpG sites under study as well as of the average (AVE) of the 7 CpG sites ± the S.E.M. Significant differences are indicated (Mann-Whitney test): * $P < 0.05$; ** $P < 0.01$.

Legend to Figure 6

Comparison of the DNA methylation status at the human *PDYN* gene promoter in the controls and alcoholic population stratified by the presence of the minor allele G for rs2235751. The bars represent the mean of the % of methylation values of individual CpG sites under study as well as of the average (AVE) of the 7 CpG sites ± the S.E.M. Significant differences are indicated (Mann-Whitney test): * $P < 0.05$; ** $P < 0.01$.

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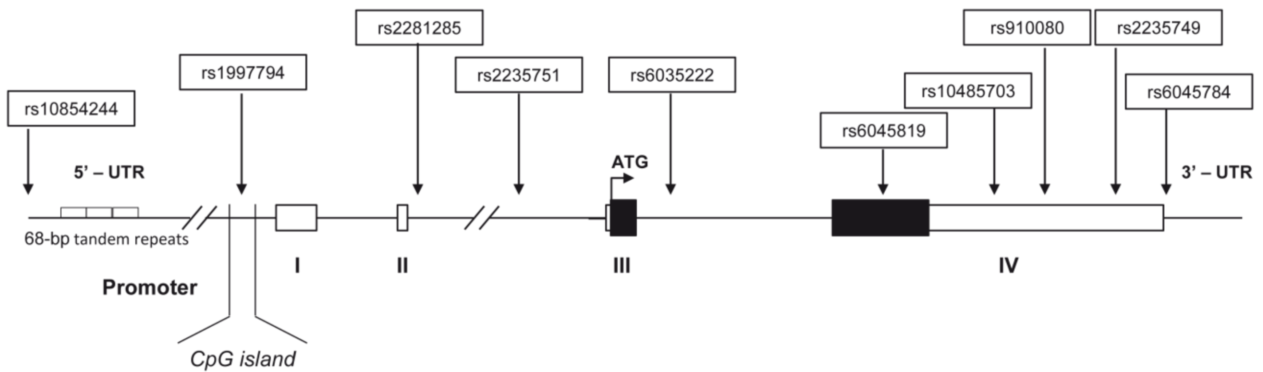
a



b

CpG island
(148 pb)

-1047 caggcaaatgtacacacaaaaagattccagcaca**cgcg**tgcaccagccaacacagagttgcc a**cg**gca**cg**caacag**cg**ggccatgca**cg**tgcctgctgact**cg**gctgtcatcttccgactctcttccgtg atcagaagactgtctcag -899

Figure 1

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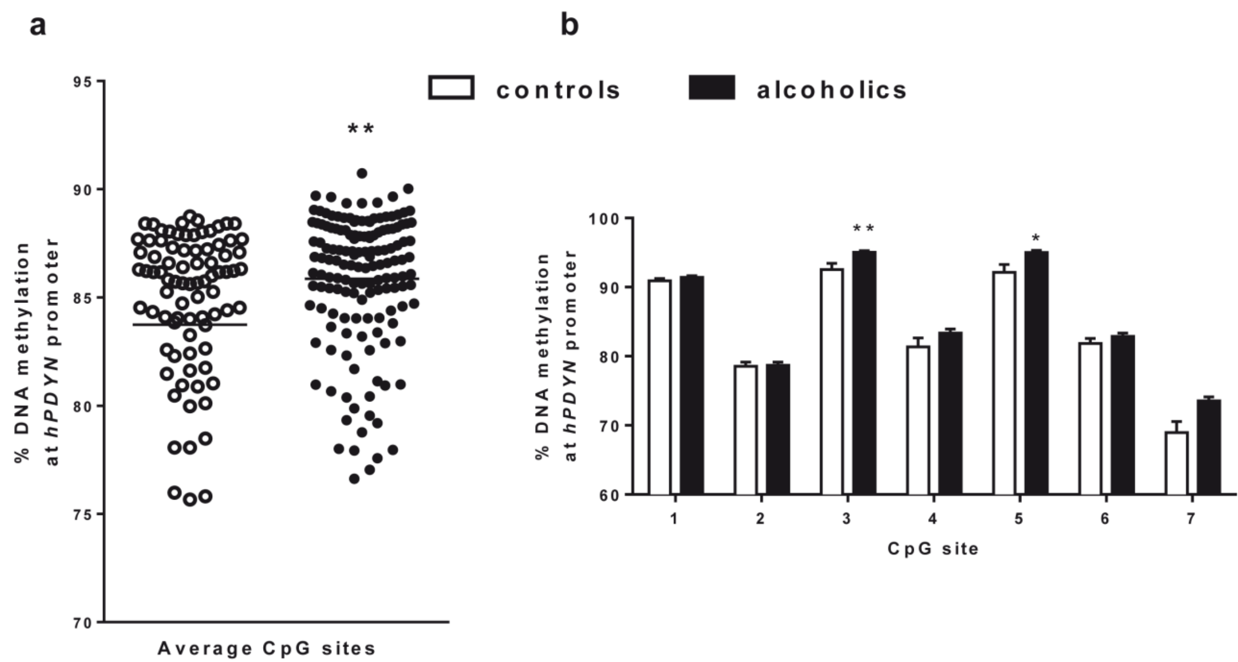

Figure 2

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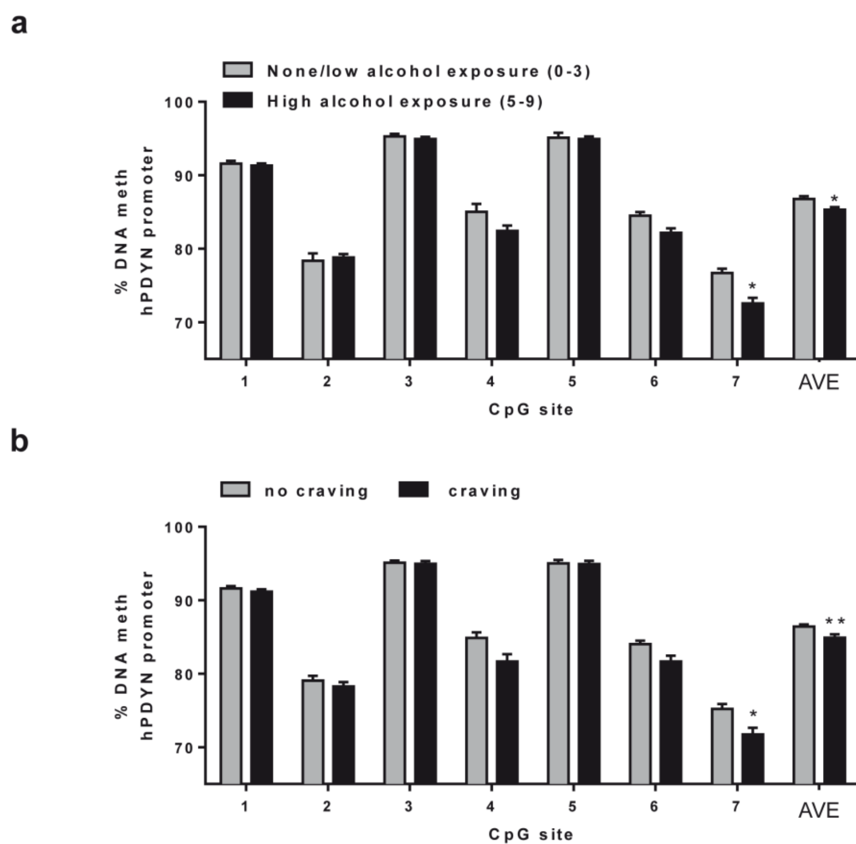

Figure 3

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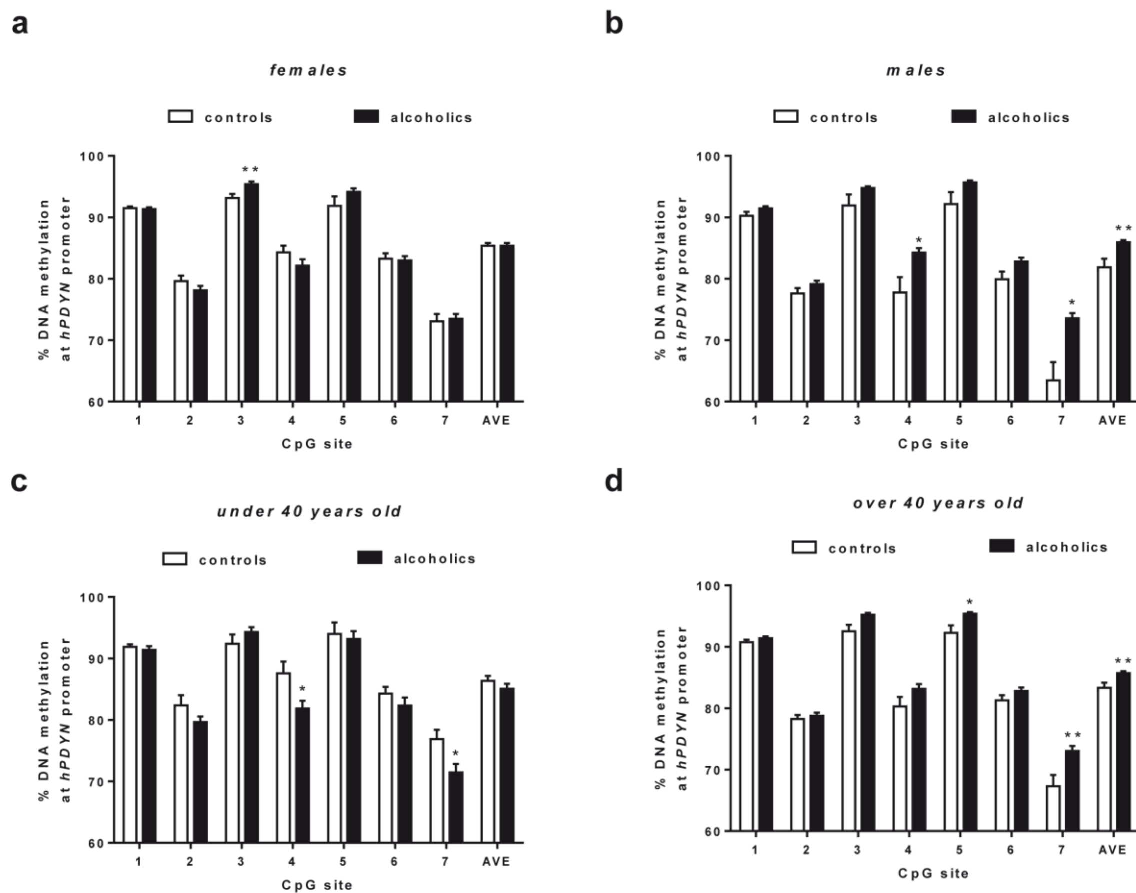

Figure 4

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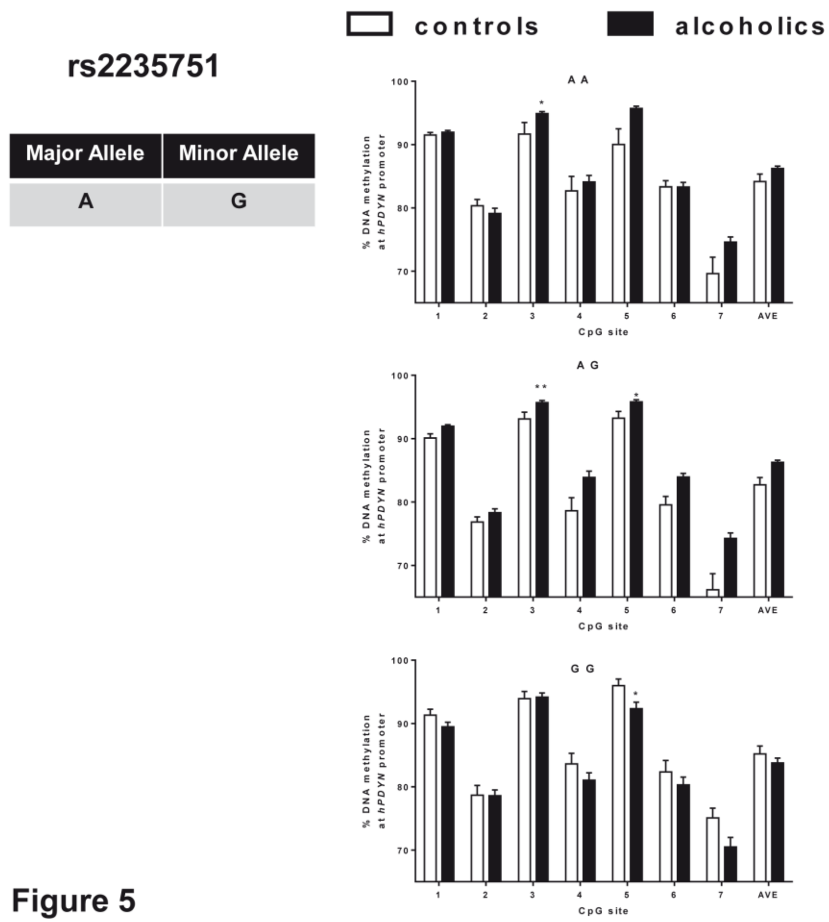

Figure 5

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G G

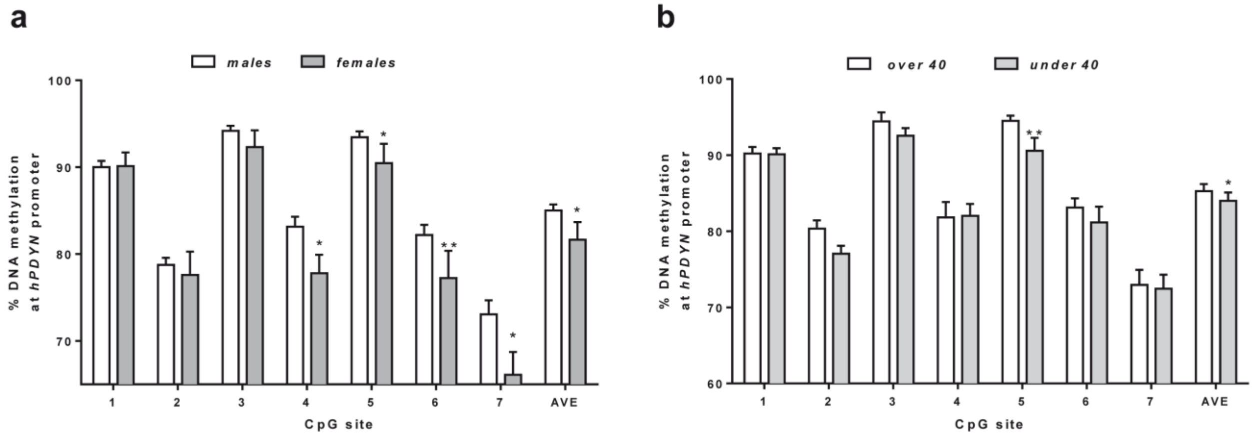

Figure 6

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Characteristic	N (%) or Mean±SD		
	All Subjects	Males	Females
Race			
European Swedish/Norwegian/Danish	744 (100%)	521 (70%)	223 (30%)
Age (years)	49.2±10.6	49.4±10.7	48.6±10.4
Drinks/day (baseline)*	12.6±5.8	12.8±6.0	12.1±5.5
Drinking days/month (baseline)	8.9±8.4	8.8±8.3	9.1±8.6
Craving	252 (33.9%)	173 (23.2%)	79 (10.6%)
Withdrawal symptoms	392(52.7%)	281 (37.8%)	111 (14.9%)
drink to relieve withdrawal	263 (67.8)	188 (48.4)	75 (19.3)
delirium and convulsion	49 (12.6)	36 (9.3)	13 (3.4)
Tolerance	202 (52 %)	149 (38.4%)	53 (13.7%)
reversed tolerance	146 (37.6)	116 (29.9)	30(7.7)
Alcohol–induced psychosis	39 (5.2%)	26 (3.5%)	13 (1.7%)
Antidepressant users	34 (8.8%	19 (4.9%)	15 (3.9%)
Antianxiety users	92 (23.7%)	48 (12.4%)	44 (11.3%)
Nicotine dependence	354 (47.5%)	201 (27.0%)	153 (20.6%)
Other substance dependence	159 (36.9%)	110 (39.7%)	49 (31.8%)
Cannabis	133 (17.9)	99 (13.3)	34 (4.6)
Cocaine	29 (3.9)	19 (2.6)	10 (1.4)
Hallucinogen	23 (3.1)	17 (2.3)	6 (0.8)
Sedatives	176 (23.7)	101 (13.6)	75 (10.1)
Stimulant	144 (19.4)	103 (13.8)	41 (5.5)
Opiate	31 (4.2)	21 (2.8)	10 (1.3)
Solvent abuse (Sniffing)	54 (7.2)	44 (5.9)	10 (1.3)
Others	160 (2.7)	140 (18.8)	20 (2.7)

**Average number of drinks per drinking day.*

Table 1 Demographic and Clinical Characteristics of the Study Sample

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SNP	Allele Major	Allele Minor		Genotype Frequency				MAF alcoholics	MAF controls	Odds ratio	*p value*
rs10854244	**A**	**T**	**TOT**	**A**	**AT**	**T**	***p value***	0,250	0,240	1,030	0,710
Chr.20:1977045			**alcoholics**	0,577	0,356	0,066	0,731				
			controls	0,579	0,364	0,057					
			FEMALES	**A**	**AT**	**T**	***p value***	0,260	0,240	1,060	0,680
			alcoholics	0,581	0,328	0,091	0,209				
			controls	0,570	0,371	0,059					
			MALES	**A**	**AT**	**T**	***p value***	0,240	0,220	1,100	0,480
			alcoholics	0,576	0,369	0,056	0,762				
			controls	0,604	0,345	0,051					
rs1997794	**T**	**C**	**TOT**	**C**	**TC**	**T**	***p value***	0,330	0,340	0,940	0,460
Chr.20:1974858			**alcoholics**	0,119	0,425	0,455	0,370				
			controls	0,114	0,461	0,425					
			FEMALES	**C**	**TC**	**T**	***p value***	0,330	0,340	0,950	0,480
			alcoholics	0,119	0,426	0,455	0,796				
			controls	0,114	0,460	0,425					
			MALES	**C**	**TC**	**T**	***p value***	0,330	0,330	1,010	0,950
			alcoholics	0,102	0,465	0,433	0,796				
			controls	0,089	0,486	0,424					
rs2281285	**T**	**C**	**TOT**	**C**	**TC**	**T**	***p value***	0,120	0,130	0,880	0,230
Chr.20: 1972460			**alcoholics**	0,022	0,197	0,781	0,241				
			controls	0,019	0,231	0,750					
			FEMALES	**C**	**TC**	**T**	***p value***	0,140	0,130	1,060	0,730
			alcoholics	0,044	0,191	0,765	**0,035**				
			controls	0,017	0,233	0,750					
			MALES	**C**	**TC**	**T**	***p value***	0,130	0,140	0,930	0,630
			alcoholics	0,030	0,196	0,774	0,605				
			controls	0,023	0,226	0,751					
rs2235751	**A**	**G**	**TOT**	**A**	**GA**	**G**	***p value***	0,260	0,240	1,140	0,090
Chr.20: 1969934			**alcoholics**	0,562	0,347	0,090	**0,036**				
			controls	0,579	0,362	0,058					
			FEMALES	**A**	**GA**	**G**	***p value***	0,220	0,240	0,890	0,390
			alcoholics	0,619	0,322	0,059	0,443				
			controls	0,575	0,370	0,055					
			MALES	**A**	**GA**	**G**	***p value***	0,250	0,240	0,810	0,140
			alcoholics	0,563	0,373	0,064	0,679				
			controls	0,591	0,342	0,066					
rs6035222	**G**	**A**	**TOT**	**A**	**GA**	**G**	***p value***	0,110	0,120	0,900	0,640
Chr.20: 1963413			**alcoholics**	0,008	0,197	0,795	**0,533**				
			controls	0,006	0,223	0,771					
			FEMALES	**A**	**GA**	**G**	***p value***	0,120	0,130	0,880	0,650
			alcoholics	0,017	0,200	0,783	**0,874**				
			controls	0,018	0,228	0,754					
			MALES	**A**	**GA**	**G**	***p value***	0,100	0,110	0,940	0,760
			alcoholics	0,004	0,200	0,796	**0,526**				
			controls	0,000	0,220	0,780					
rs6045819	**A**	**G**	**TOT**	**A**	**AG**	**G**	***p value***	0,100	0,110	0,830	0,540
Chr.20: 1961134			**alcoholics**	0,815	0,177	0,008	0,543				
			controls	0,782	0,207	0,011					
			FEMALES	**A**	**AG**	**G**	***p value***	0,100	0,130	0,710	0,240
			alcoholics	0,817	0,174	0,009	0,458				
			controls	0,765	0,209	0,026					
			MALES	**A**	**AG**	**G**	***p value***	0,100	0,110	0,890	0,580
			alcoholics	0,816	0,176	0,008	0,605				
			controls	0,790	0,206	0,004					
rs10485703	**A**	**G**	**TOT**	**A**	**AG**	**G**	***p value***	0,090	0,110	0,810	0,230
Chr.20: 1960313			**alcoholics**	0,829	0,163	0,008	0,474				
			controls	0,794	0,195	0,011					
			FEMALES	**A**	**AG**	**G**	***p value***	0,090	0,130	0,700	0,230
			alcoholics	0,826	0,165	0,009	0,455				
			controls	0,774	0,200	0,026					
			MALES	**A**	**AG**	**G**	***p value***	0,090	0,100	0,860	0,500
			alcoholics	0,833	0,159	0,008	0,529				
			controls	0,803	0,193	0,004					
rs910080	**A**	**G**	**TOT**	**A**	**AG**	**G**	***p value***	0,230	0,270	0,800	0,070
Chr.20: 1960226			**alcoholics**	0,588	0,365	0,047	**0,175**				
			controls	0,522	0,413	0,064					
			FEMALES	**A**	**AG**	**G**	***p value***	0,240	0,270	0,840	0,420
			alcoholics	0,583	0,357	0,061	**0,341**				
			controls	0,570	0,316	0,114					
			MALES	**A**	**AG**	**G**	***p value***	0,220	0,270	0,770	0,080
			alcoholics	0,591	0,372	0,036	**0,126**				
			controls	0,500	0,459	0,041					
rs2235749	**A**	**G**	**TOT**	**G**	**AG**	**A**	***p value***	0,240	0,280	0,820	0,090
Chr.20: 1959939			**alcoholics**	0,050	0,377	0,573	0,215				
			controls	0,064	0,426	0,510					
			FEMALES	**G**	**AG**	**A**	***p value***	0,240	0,290	0,800	0,290
			alcoholics	0,061	0,365	0,574	0,352				
			controls	0,115	0,345	0,540					
			MALES	**G**	**AG**	**A**	***p value***	0,230	0,260	0,840	0,230
			alcoholics	0,041	0,380	0,580	0,128				
			controls	0,029	0,469	0,502					
rs6045784	**T**	**C**	**TOT**	**C**	**TC**	**T**	***p value***	0,090	0,110	0,810	0,230
Chr.20: 1956663			**alcoholics**	0,008	0,163	0,829	0,474				
			controls	0,011	0,195	0,794					
			FEMALES	**C**	**TC**	**T**	***p value***	0,090	0,130	0,700	0,230
			alcoholics	0,009	0,165	0,826	0,455				
			controls	0,026	0,200	0,774					
			MALES	**C**	**TC**	**T**	***p value***	0,090	0,100	0,860	0,500
			alcoholics	0,008	0,159	0,833	0,530				
			controls	0,004	0,193	0,803					

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Highlights

- Altered DNA methylation in prodynorphin gene promoter in human alcoholics.
- Selected SNP associated with alcoholism and altered DNA methylation.
- Relevant for biomarkers prediction of disease trajectories and therapeutic outcome.

ACCEPTED MANUSCRIPT