Genome-wide DNA methylation analysis of heavy cannabis use in a New Zealand longitudinal cohort.

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ABSTRACT

Heavy, long term cannabis use is associated with an increased risk of adverse psychosocial outcomes, depression and schizophrenia. Here we examined the effect of cannabis use, with and without tobacco, on genome-wide DNA methylation in a longitudinal birth cohort (Christchurch Health and Development Study). We found the most differentially methylated sites in cannabis with tobacco users were in the *AHRR* and *F2RL3* genes, replicating previous studies on the effects of tobacco. Cannabis-only users had no evidence of differential methylation in these genes, or at any other loci at the epigenome-wide significance level ($P<10^{-7}$). However, there were 521 sites differentially methylated at $P<0.001$ which were enriched for genes involved in cardiomyopathy and neuronal signalling. Further, the most differentially methylated loci were associated with genes whose function is consistent with the psychosocial outcomes associated with cannabis use (e.g. *TMEM190*, *MUC3L*, *CDC20* and *SP9*). We conclude that the effects of cannabis use on the mature human blood methylome differ from the effects of tobacco use, are less pronounced, and will require larger sample sizes in order for specific loci to be identified.

Introduction

Cannabis use is an important global public health issue, and a growing topic of controversy and debate [1; 2]. It is the most widely used illicit psychoactive substance in the world [3], and the potential medicinal and therapeutic benefits of cannabis and its main active ingredients tetrahydrocannabinol (THC) and cannabidiol (CBD) are gaining interest [4-6]. There is strong evidence to suggest that the heavy and prolonged use of cannabis may be associated with increased risk of adverse outcomes in a number of areas, including mental health (psychosis [7-9], schizophrenia[10; 11], depression [12; 13]), impaired educational achievement [14], and abuse of other illicit drugs [15].

Drug metabolism, drug response and drug addiction have strong genetic components [16], and multiple genome-wide association studies (GWAS) have identified genes and allelic variants that are likely contributors to substance use disorders [17; 18]. Furthermore, there are patterns of cannabis use disorder that are heritable, with some users progressing to drug dependency [19-22], implying that there are genetic as well as environmental influences on cannabis use and addiction [22]. As such, several candidate loci for complex phenotypes such as lifetime cannabis use have recently been identified [3; 23] that explain a proportion of the variance in cannabis use heritability. However, complex phenotypes such as drug response and addiction are influenced by multiple loci, each of which usually has a small individual effect size [24], and such loci are frequently located in non-coding regions of the genome [25; 26]. Therefore, it is clear that biological responses to substances of abuse, such as cannabis, are determined by more than direct allelic variants [27].

Epigenetic modifications of DNA, such as DNA methylation, may contribute to complex phenotypes [28]. Methylation of cytosine residues within CpG dinucleotides is an important mechanism of

variation and regulation in the genome [29; 30 31 32]. Cytosine methylation, particularly in the promoter region of genes, is generally associated with a decrease in transcription [33], and there exists a correlation in DNA methylation in the first intron and gene expression, conserved across tissues and vertebrate species [34]. Modulation of methylation at CpG sites within the human genome can result in an epigenetic pattern that is specific to individual environmental exposures, and may contribute to disease [35-38]. For example, environmental factors such as drugs, alcohol, stress, nutrition, bacterial infection, and even exercise [37; 39-42] have been associated with methylation changes, and differentially methylated regions (DMRs) have been identified that are specifically modified in tobacco smokers versus non-smokers [43-45]. Some of these methylation changes have been shown to endure, inducing lasting biological changes, with potentially adverse health outcomes [37]. Conversely, some methylation changes are dynamic and transient. For example, alcohol consumption affects genome-wide methylation patterns in a severity-dependent manner [46] and some of these widespread changes revert upon abstinence from alcohol consumption [47]. In addition, genome-wide levels of methylaton in former tobacco smokers depend on cessation time, eventually reaching levels close to those who had never smoked tobacco [48]. Thus, not only can methylation indicate a particular environmental exposure, but importantly, surveying methylation changes may shed light on the dynamic interaction between the environment and the genome, and reveal novel pathways involved in the biological response to environmental factors.

The impact of environmental factors such as recreational drug use has been associated with adverse psychosocial outcomes, particularly in youth [49-53]. Associations such as these have been shown to be mediated by interactions with the genome [54; 55], hence it is possible that epigenetic modification could mediate the association between drug use and adverse psychosocial outcomes. Therefore, we sought to determine whether regular cannabis users displayed differential cytosine methylation compared to non-cannabis users. Cannabis users in this study are participants from the Christchurch

Health and Development Study (CHDS). The CHDS is a longitudinal study of a birth cohort of 1265 children born in 1977 in Christchurch, New Zealand, who have been studied on 24 occasions from birth to the age of 40 (n=987 at age 30, with blood collected at approximately age 28). In the early 1990s, research began into the initiation and consequences of cannabis use amongst CHDS participants; cannabis use was assessed prospectively over the period up to the collection of DNA, and this research now spans the period from mid-adolescence to adulthood, with data on dependence, educational achievement, psychotic symptoms, illicit drug use, depression, suicidal behaviours and driver risks collected over this period. Unusually, the CHDS cohort contains a subset of cannabis users who have never consumed tobacco, enabling the investigation of the specific effects of cannabis consumption, in isolation, on DNA methylation in the human genome.

Methods

Cohort and study design

Between the ages of 28 to 30, CHDS participants were approached to provide a peripheral blood sample for DNA analysis. A subset of the >800 participants who consented and provided a blood sample was used in the present study, comprising a total of 96 participants. Cases (regular cannabis users, n = 48) were matched with controls (n = 48) for sex, ethnicity and family of origin socioeconomic status (Table 1). Case participants were partitioned into two subsets: one that contained cannabis-only users (who had never consumed tobacco, "cannabis-only", n = 24), and one that contained cannabis users who also consumed tobacco ("cannabis with tobacco", n = 24) and were selected on the basis that they either met DSM-IV [56] diagnostic criteria for cannabis dependence, or had reported using cannabis on a daily basis for a minimum of three years prior to age 28. The mode of cannabis consumption was via smoking, for all participants. The median

duration of regular use for selected cases was 9 years (range 3-14 years). Control participants had never used cannabis or tobacco. Additionally, there are comprehensive SNP data available on all participants [57]. All aspects of the study were approved by the Southern Health and Disability Ethics Committee, under application number CTB/04/11/234/AM10 "Collection of DNA in the Christchurch Health and Development Study", and the CHDS ethics approval covering collection of cannabis use: "16/STH/188/AM03 The Christchurch Health and Development Study 40 Year Follow-up".

DNA extraction and methylation arrays

DNA was extracted from whole blood using the KingFisher Flex System (Thermo Scientific, Waltham, MA USA), as per the published protocols. DNA was quantified via NanoDrop™ (Thermo Scientific, Waltham, MA USA) and standardised to 100ng/µl. Equimolar amounts were shipped to the Australian Genomics Research Facility (AGRF, Melbourne, VIC, Australia) for analysis with the Infinium® MethylationEPIC BeadChip (Illumina, San Diego, CA USA).

Bioinformatics and Statistics

All analysis was carried out using R (Version 3.5.2 [58]). Prior to normalisation, quality control was performed on the raw data. Firstly, sex chromosomes and 150 failed probes (detection P value > 0.01 in at least 50% of samples) were excluded from analysis. Furthermore, potentially problematic probes with adjacent SNVs or that did not map to a unique location in the genome [59] were also excluded, leaving 700,296 CpG sites for further analysis. The raw data were then normalised with the NOOB procedure in the minfi package [60] (Supplementary Figure 1). Normalisation was checked by visual inspection of intensity densities and the first two components from Multi-Dimensional Scaling of the 5000 most variable probes (Supplementary Figures 2 and 3). The proportions of cell types (CD4+, CD8+ T cells, Natural Killer, B cells, Monocytes and Granulocytes) in each sample were

estimated with the Flow.Sorted.Blood package [61]. Linear models were fitted to the methylated/unmethylated or M ratios using limma [62]. Separate models were fitted for cannabis-only vs. controls, and cannabis plus tobacco users vs. controls. Both models contained covariates for sex (bivariate), socioeconomic status (three levels), batch (bivariate), population stratification (four principal components from 5000 most variable SNPs) and cell type (five continuous). β values were calculated, defined as the ratio of the methylated probe intensity (M) / the sum of the overall intensity of both the unmethylated probe (U) + methylated probe (M). P values were adjusted for multiple testing with the Benjamini and Hochberg method and assessed for genomic inflation with bacon [63]. Differentially methylated CpG sites were matched to the nearest neighbouring genes in Hg19 using GRanges [64], and their official gene symbols were tested for enrichment in KEGG 2019 human pathways with EnrichR [65].

Results

Data normalisation

Modelled effects showed no indication of genomic inflation with λ =1.04 for cannabis-only users (Supplementary Figure 4a) and λ = 0.855 for cannabis with tobacco users (Supplementary Figure 4b), versus controls. These were confirmed with bacon for cannabis-only (inflation = 0.98, bias = 0.044) and cannabis with tobacco users (inflation = 0.91, bias = 0.19). Inflation values less than 1 suggest that the results may be conservative.

Cannabis with tobacco users had a significantly lower proportion of natural killer cells than controls (1.8%, 0.4% - 3.2%, P<0.014) with no other proportions differing significantly. After adjusting for

multiple comparisons this was not significant (P=0.08) however we note that it is consistent with other findings that NK-cells are suppressed in the plasma of tobacco smokers [66; 67].

Differential methylation

The most differentially methylated CpG sites for cannabis users relative to controls differ in the absence of tobacco (Table 2) and presence of tobacco smoking (Table 3). There were five individual CpG sites which were significantly differentially methylated after adjustment for multiple testing between users and controls when cannabis with tobacco was used (Table 3 and Figure 1). The top CpG sites in the *AHRR, ALPG* and *F2RL3* genes (Table 2) are consistent with previous studies on tobacco use without cannabis (e.g. [43; 48; 68; 69]), and cg17739917 is in the same CpG-island as other CpGs previously shown to be hypomethylated in response to tobacco. Cannabis-only users showed no CpG sites differentially methylated after correction for multiple testing (Figure 2), however the most differentially methylated site was hypermethylation of cg12803068 in the gene *MYO1G*, which has been found to be hypermethylated in response to tobacco use in previous studies [68].

To describe the data we chose a nominal P value of 0.001 and found that at this level both cannabis-only and cannabis with tobacco users showed relatively higher rates of hypermethylation than hypomethylation and similar locations with respect to annotated features (Table 4). There were two sites hypermethylated in both cannabis-only and cannabis with tobacco users; cg02514528, in the promoter of *MARC2*, and cg27405731 in *CUX1*, while cg26542660 in the promoter of *CEP135* was hypomethylated in both user groups. The second most differentially methylated site in cannabis-only users was cg02234936 in the *ARHGEF1* gene and this was hypermethylated in the cannabis with tobacco users.

Pathway enrichment analyses

We then took the genes containing differentially methylated probes at P<0.001 for the cannabis-only group that were within or near genes (that is, not up or downstream in Table 4) and compared them with human KEGG pathways using Enrichr. The hypermethylated CpG sites (n = 420) showed enrichment in the arrhythmogenic right ventricular cardiomyopathy pathway at an Enrichr adjusted P value of 0.03 and enrichment in the glutamatergic synapse and long term potentiation pathway at P=0.05 (Figure 3). Enrichment analysis of hypomethylated loci (n = 101) in cannabis-only users did not identify any KEGG pathways at or near adjusted significance of 0.05 (Figure 4).

Discussion

Many countries have recently adopted, or are considering, lenient polices regarding the personal use of cannabis [70-72]. This approach is supported by the evidence that the prohibition of cannabis can be harmful [73]. Further, the therapeutic benefits of cannabis are gaining traction, most recently as an opioid replacement therapy [74]. However, previous studies, including analyses of our study cohort, have reported an association between cannabis use and negative psychosocial outcomes, most strongly in youth [75; 76]. Establishing the use of cannabis exclusive of tobacco is complicated by the widespread practice of mulling or mixing cannabis with tobacco, a practice not common in New Zealand, and also the biases associated with self-reporting use of an illicit substance. The CHDS prospectively and confidentially assessed recreational substance use at multiple time points during the life course of participants, thus reducing recall and related biases. Our study was limited by sample size achieving approximately 10% power at $P=10^{-7}$ to detect the largest standardized effect size found.

Here we investigated whether changes in an epigenetic mark, DNA methylation, were altered in cannabis users, versus controls. Consistent with previous reports, we observed greatest differential methylation in cannabis with tobacco users in the *AHRR* and *F2RL3* genes, replicating previous studies on the epigenetic effects of tobacco [43; 48; 68; 69]. These changes, however, were not apparent in the cannabis-only data. Further, only two nominally significantly differentially methylated CpG sites were observed in both the cannabis-only and cannabis with tobacco analyses. Taken together, this suggests that tobacco use dominates any effects of cannabis on the human blood methylome, and that these data are not completely confounded by the inclusion of cannabis users. The strength of the tobacco signature in the mixed dataset suggests that a cautious approach should be adopted when interpreting studies of DNA methylation in cannabis users that do not, or cannot, exclude tobacco smokers. Interestingly, the two nominally significant CpG sites that overlap between the cannabis-only and the cannabis with tobacco data are located within the *MARC2* and *CUX1* genes. A SNP in *MARC2* has been provisionally associated with the biological response to antipsychotic therapy in schizophrenia patients [77], and the *CUX1* gene has an established role in neural development and contains variants associated with developmental delay [78], phenotypes which are relevant to sequelae of both tobacco and cannabis use.

Cannabis affects the brain, leading to perceptual alterations, euphoria and relaxation [19]. However, prolonged use is associated with a myriad of mood disorders, including adult psychosis [7; 8; 53; 79; 80], mania [13], personality disorders [21], reduced educational achievement [14] and depression [12]. We did not detect significantly differentially methylated loci associated with exclusive cannabis use at the epigenome-wide level. However, an assessment of those top loci reaching nominal significance identified CpG sites within genes involved in brain function and mood disorders. For example, the top cannabis-only probes are within or nearest to (that is no intervening coding region) genes such

as *MUC3L* (associated with autism, ADHD, bipolar disorder, major depressive disorder, schizophrenia [81; 82]), *CDC20* (bipolar disorder, cognitive function, nervous system development [83]), *DUS3L* (risk-taking behaviour [84]), *TMEM190* (unipolar depression [85]), *FOXB1* (interacts with *TLE3* and *CTNNB1* which have associations with cognitive performance [86-88]), *KIAA1324L/GRM3* (schizophrenia, autism [82; 89-94]), *DDX25* (age of onset of Alzheimer's disease, autism, ADHD, bipolar disorder, major depressive disorder, schizophrenia [81; 95; 96]), *TNRC6B* (PI3K/Akt signalling pathway, long-term potentiation [97; 98]) and *SP9* (educational attainment [99]).

Pathway enrichment revealed that hypermethylation in cannabis users who did not use tobacco was over-represented in genes associated with cardiomyopathies and neural signalling. This is consistent with the global clinical concern around cardiovascular complications of cannabis use, particularly in low-risk youth [100], and the debate about the effect of cannabis on the heart, with many clinicians urging caution [101-103]. Further, many genes associated with neural signalling pathways contain variants that are now well established to be associated with psychiatric and neurodevelopmental disorders [104; 105], and hence are consistent with early findings that higher use of cannabis is associated with higher incidence of psychotic symptoms and poorer outcomes in the CHDS[7; 12; 14; 52; 53; 73; 106; 107]. Interestingly, hypermethylation in cannabis-only users is enriched for genes in the glutamatergic synapse which is consistent with recent discoveries of exonic SCZ risk variants in this pathway, although we note our top loci was in a different subunit of the NMDA receptor [108]. Therefore, while nominal, the associations presented are consistent with the proven psychosocial impacts of cannabis. While we have not implicated any gene at the genome-wide significance level, this by no means precludes a role for DNA methylation in the biological response to cannabis, and the data suggest that further investigations in larger cohorts are warranted.

While tobacco use has declined on the back of state-sponsored cessation programs [109], the high rate of cannabis use in New Zealand and globally is a significant issue [110]. Cannabis use disorder is growing in prevalence [111], and regular or heavy cannabis use has been associated with increased risk of using other illicit drugs, abusing or becoming depending on other illicit drugs, and using a wider variety of other illicit drugs [15; 112; 113]. Understanding the epigenetic effects of cannabis will contribute to the growing literature into quantifying the biological effects of heavy cannabis use, and also may aid in developing strategies to mitigate cannabis use and abuse. Thus, in conjunction with the current increase in the decriminalisation or legalisation of cannabis use for therapeutic and/or recreational purposes, further studies are required to elucidate the long-term effects of cannabis use on both the epigenome and the developing and mature human brain.

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Table 1 - Christchurch Health and Development Study (CHDS) participants selected for EPIC arrays. Cases and controls were matched as closely as possible by sex, ethnicity and parental socioeconomic status/occupation. Cases are comprised of regular cannabis users, half of whom have never used tobacco. Controls are comprised of individuals with no exposure to cannabis or tobacco. 'Other' ethnicity is a combination of Māori and Pacific Island participants.

Table 2 – Association of cannabis and DNA methylation in the absence of tobacco (cannabis-only users). Most significantly differentially methylated sites between users and controls after adjustment for sex, socioeconomic status, population genetics and cell counts. Beta values with P values, nominal and adjusted by the Benjamini and Hochberg method to control for multiple comparisons. Locations are relative to hg19 with gene names for overlapping genes or nearest 5' gene with distance to the 5' end shown.

Table 3 Association of cannabis and DNA methylation in the presence of tobacco (cannabis with tobacco users). Most significantly differentially methylated sites between users and controls after adjustment for sex, socioeconomic status, population genetics and cell counts. Beta values with P values, nominal and adjusted by the Benjamini and Hochberg method to control for multiple comparisons. Locations are relative to hg19 with gene names for overlapping genes or nearest 5' gene with distance to the 5' end shown.

Table 4. Counts of CpG sites from cannabis-only and cannabis with tobacco users vs. non-users. Counts of significant sites at a nominal P value of 0.001 and at a Benjamini and Hochberg adjusted P value < 0.05. 'Both' indicates the number of probes of each type that are present and shared across both the cannabis only and cannabis with tobacco datasets.

Figure 1 – A Manhattan plot of the genome-wide CpG sites found in association with cannabis and tobacco smoking in the sub-selected CHDS cohort analysed using the Illumina EPIC array system. The CpG sites are plotted as -log10(p) values with the most significant methylated sites labelled with the gene the CpG site resides in.

Figure 2 - A Manhattan plot of the genome wide CpG sites found in association with cannabis-only users, compared to controls using Illumina EPIC array system. The CpG sites are plotted as -log10(p) values with the most nominally significant different methylated sites labelled with the gene the CpG site resides in.

Figure 3 – Genetic networks associated with hypermethylation in cannabis-only users. Pathways from KEGG 2019 Human identified by Enrichr. Genes shown by filled cells are hypermethylated in cannabis-only users and included in named pathway.

Figure 4 – Genetic networks associated with hypomethylation in cannabis-only users. Pathways from KEGG 2019 Human identified by Enrichr. Genes shown by filled cells are hypomethylated in cannabis-only users and included in named pathway.

Table 1

		Cases	Controls
Sex	Male	37	37
	Female	11	11
Ethnicity	European	35	45
	Other	13	3
Socioeconomic status	Professional/managerial	6	6
	Clerical/technical/skilled	21	21
	Semi-skilled/unskilled	21	21

Table 2

CpG	Gene	Location	Distance	Cannabis	Control	Difference	P value	P value
				β_U	β_C	$\beta_U - \beta_C$	*Nominal*	*Adjusted*
cg12803068	MYO1G	intron		0.8	0.71	0.1	6.30E-07	0.4
cg02234936	ARHGEF1	intron		0.14	0.13	0.01	1.10E-06	0.4
cg01695406	TMEM190	intron		0.82	0.77	0.05	3.00E-06	0.6
cg24875484	MUCL3	intron		0.1	0.09	0.01	3.90E-06	0.6
cg05009104	MYO1G	intron		0.79	0.74	0.05	5.90E-06	0.6
cg00470351	CDC20	exon		0.4	0.38	0.02	6.10E-06	0.6
cg24060040	DUS3L	upstream	11,018	0.11	0.08	0.03	6.30E-06	0.6
cg12322720	FOXB1	downstream	150,921	0.58	0.52	0.06	8.90E-06	0.7
cg16746471	KIAA1324L	promoter	374	0.1	0.08	0.02	1.10E-05	0.7
cg04180046	MYO1G	intron		0.56	0.52	0.04	1.20E-05	0.7
cg06955687	DDX25	downstream	28,769	0.74	0.7	0.04	1.20E-05	0.7
ch.22.70704	TNRC6B	downstream	159,737	0.06	0.04	0.01	1.30E-05	0.7
cg09344183	SP9	downstream	5,964	0.06	0.05	0.01	1.40E-05	0.7
cg06693983	TMEM190	exon		0.84	0.76	0.08	1.40E-05	0.7
cg26069230	ADAP2	exon		0.16	0.14	0.01	1.50E-05	0.7

Table 3

CpG	Gene	Location	Distance	Cannabis	Control	Difference	P value	P value
				β_U	β_C	$\beta_U - \beta_C$	*Nominal*	*Adjusted*
cg05575921	AHRR	intron		0.66	0.89	-0.24	1.40E-11	0.00001
cg21566642	ALPG	downstream		0.44	0.62	-0.17	9.90E-11	0.00003
cg03636183	F2RL3	exon		0.59	0.68	-0.09	2.60E-09	0.0006
cg01940273	ALPG	downstream		0.53	0.63	-0.09	3.60E-08	0.00636
cg17739917	RARA	intron		0.37	0.47	-0.1	5.60E-08	0.00783
cg01541424	LINC02393	upstream	491,508	0.17	0.13	0.04	6.30E-07	0.07
cg12828729	TIFAB	upstream	35,880	0.56	0.5	0.06	7.10E-07	0.07
cg10148067	MTFR1	upstream	3,928	0.91	0.88	0.02	7.70E-07	0.07
cg14391737	PRSS23	intron		0.36	0.42	-0.06	9.60E-07	0.07
cg07219494	TENM2	upstream	303,359	0.7	0.75	-0.05	1.40E-06	0.1
cg05723029	PIEZO2	intron		0.83	0.79	0.05	1.50E-06	0.1
cg03329539	ALPG	downstrea	11,777	0.36	0.41	-0.05	3.20E-06	0.2
cg24994593	LDLRAD3	intron		0.9	0.89	0.02	4.20E-06	0.2
cg25009999	LINC01168	downstrea	14,152	0.93	0.92	0.01	5.60E-06	0.3
cg13957017	TTLL6	intron		0.72	0.69	0.03	7.30E-06	0.3

Table 4

	Cannabis only		Tobacco + Cannabis		Both
Differentially Methylated loci (FWER = 0.05)	0		6		
Differentially Methylated loci (P<0.001)					
Total	521		533		
Hypermethylated	420	80.6%	403	75.6%	2
Hypomethylated	101	19.4%	130	24.4%	1
Hyper (Cannabis) Hypo (Cannabis + Tobacco)					1
Location					
Intron	216	41.5%	264	49.5%	
Exon	97	18.6%	65	12.2%	
Exon Boundary	0		0		
Promoter	89	17.1%	60	11.3%	
3' UTR	3	0.6%	1	0.2%	
5' UTR	0		0		
3' (downstream)	62	11.9%	76	14.3%	
5' (upstream)	54	10.4%	67	12.6%	

Figure 1

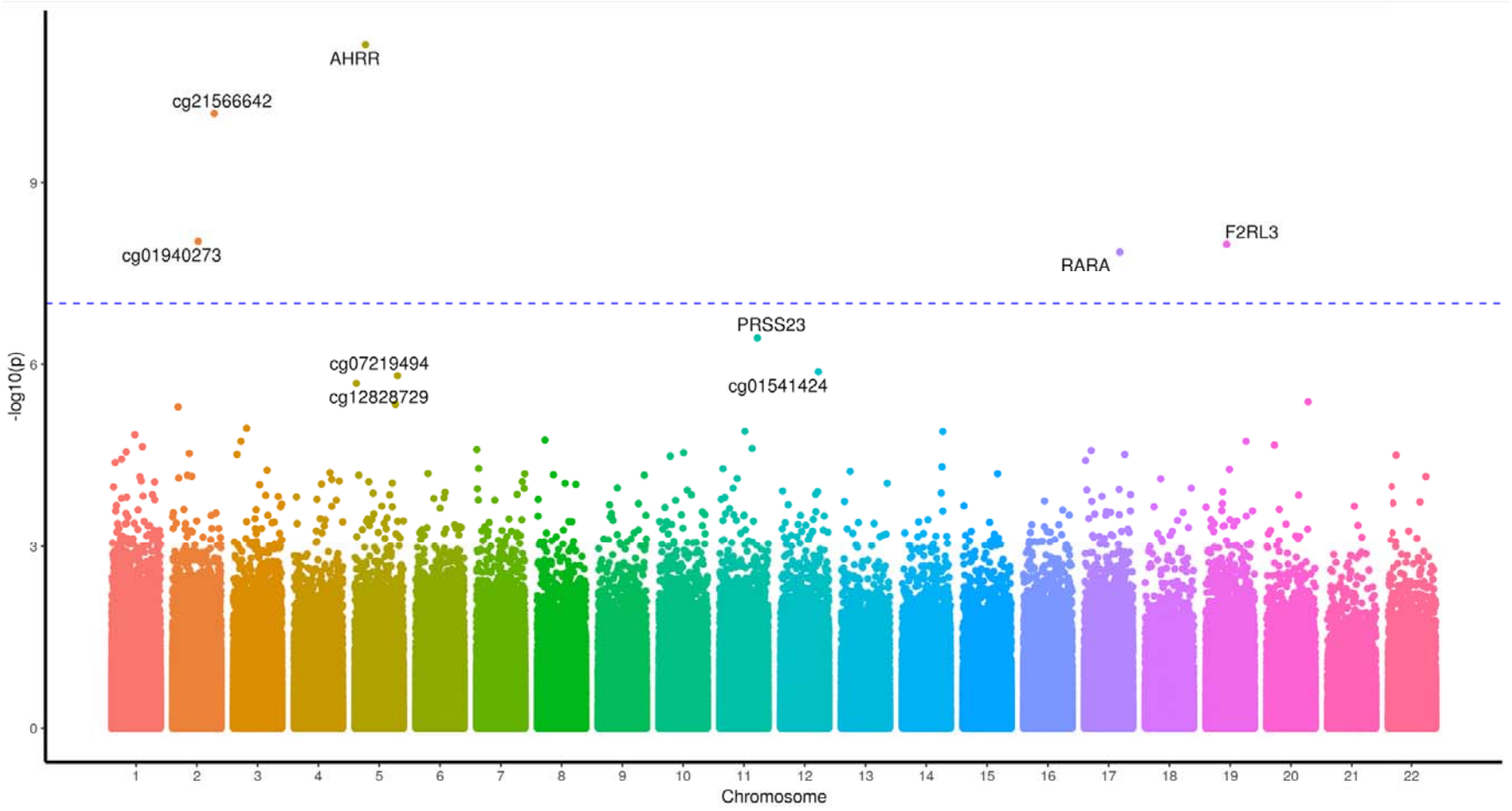

Figure 2

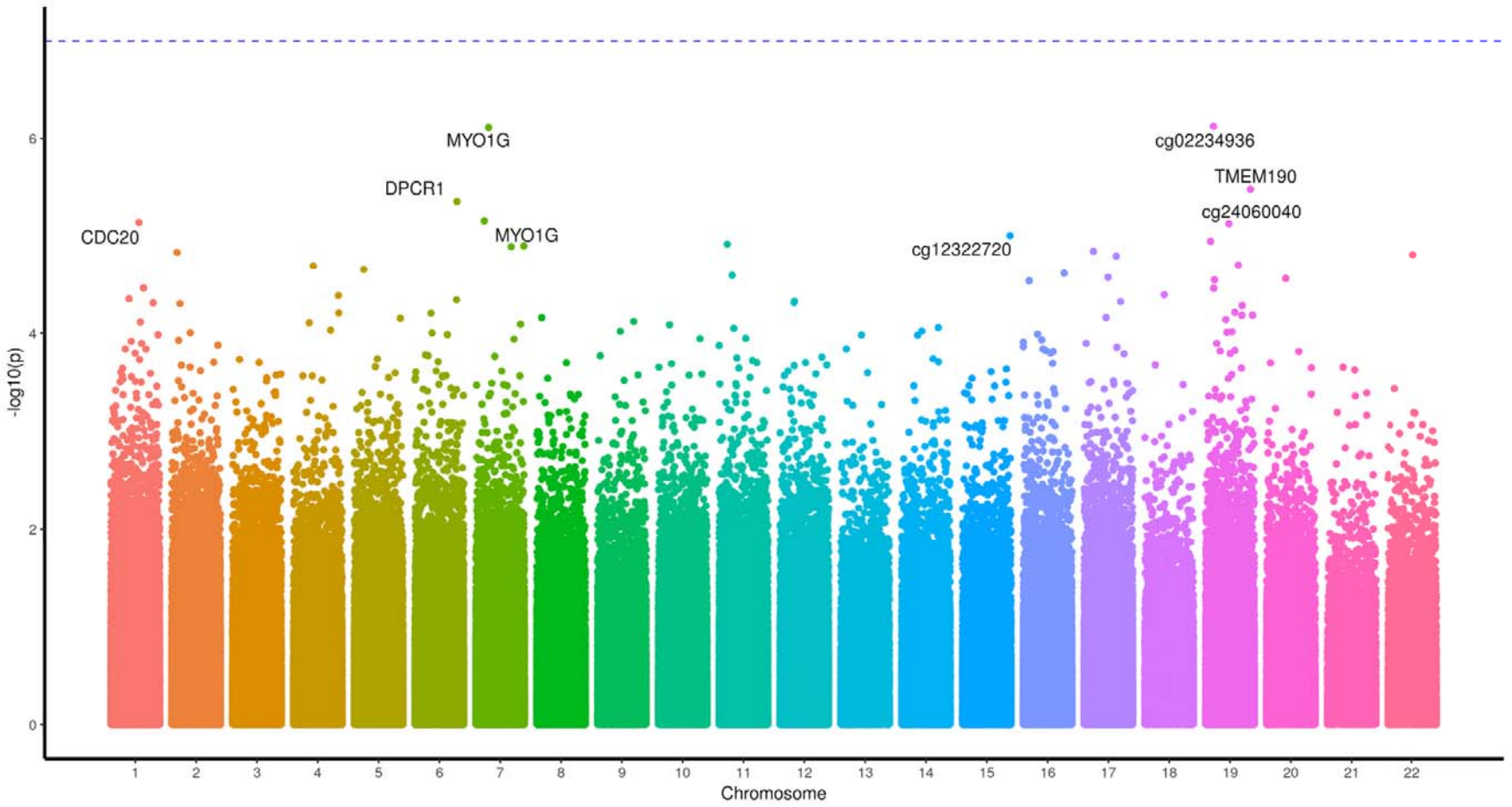

Figure 3

Pathway	P value	adjusted P value	Score
Arrhythmogenic right ventricular cardiomyopathy	0.0001	0.03	58.9
Glutamatergic synapse	0.0003	0.05	37.1
Long-term potentiation	0.0005	0.05	44.8
cAMP signaling pathway	0.0015	0.11	20.3
Adrenergic signaling in cardiomyocytes	0.0016	0.10	23.4
Hypertrophic cardiomyopathy (HCM)	0.0018	0.09	29.5
Dilated cardiomyopathy (DCM)	0.0026	0.11	26.0
Nicotine addiction	0.0030	0.12	38.4
Dopaminergic synapse	0.0038	0.13	19.7
Type II diabetes mellitus	0.0051	0.16	30.5

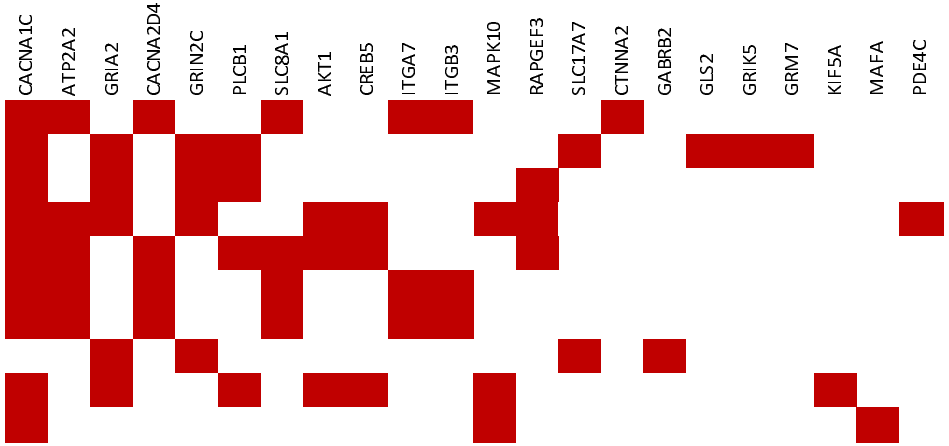

Figure 4.

Pathway	P value	adjusted P value	Score
JAK-STAT signaling pathway	0.02	1	20.9
Epithelial cell signaling in Helicobacter pylori infection	0.02	1	31.4
Adipocytokine signaling pathway	0.02	1	30.8
Human papillomavirus infection	0.03	1	12.3