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

Amy J. Osborne,[1,6]* John F. Pearson,[2,6] Alexandra J. Noble,[1] Neil J. Gemmell,[3] L. John Horwood,[4] Joseph M. Boden,[4] Miles Benton,[5] Donia P. Macartney-Coxson,[5] Martin A. Kennedy[2]**

1 School of Biological Sciences, University of Canterbury, Christchurch 8041, New Zealand

2 Department of Pathology and Biomedical Science, University of Otago Christchurch, Christchurch 8011, New Zealand

3 Department of Anatomy, Otago School of Medical Sciences, University of Otago, Dunedin 9054, New Zealand

4 Department of Psychological Medicine, University of Otago Christchurch, Christchurch 8011, New Zealand

5 Human Genomics, Institute of Environmental Science and Research, Kenepuru Science Centre, Porirua 5240, New Zealand

6 These authors contributed equally to this work

Correspondence

* Amy Osborne, amy.osborne@canterbury.ac.nz

** Martin Kennedy, martin.kennedy@otago.ac.nz

ABSTRACT

Cannabis use is of increasing public health interest globally. 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 with reported roles in brain function (e.g. *TMEM190*, *MUC3L*, *CDC20* and *SP9*). We conclude that the effects of cannabis use on the mature human blood methylome differ from, and are less pronounced than, the effects of tobacco use, and that larger sample sizes are required to investigate this further.

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]), and illicit drug abuse [14].

Drug metabolism, drug response and drug addiction have known genetic components [15], and multiple genome-wide association studies (GWAS) have identified genes and allelic variants that are likely contributors to substance use disorders [16; 17]. There are aspects of cannabis use disorder that are heritable [18-21], and several candidate loci for complex phenotypes such as lifetime cannabis use have recently been identified [3; 22] that explain a proportion of the variance in cannabis use heritability. Complex phenotypes like these are influenced by multiple loci, each of which usually has a small individual effect size [23], and such loci are frequently located in non-coding regions of the genome [24; 25], making their biological role difficult to elucidate.

Epigenetic mechanisms are involved in the interaction between the genome and environment; they respond to changes in environmental stimuli (such as diet, exercise,

drugs), and act to alter chromatin structure and thus regulate gene expression [26]. Epigenetic modifications, such as DNA methylation, contribute to complex traits and diseases [27; 28]. Methylation of cytosine residues within CpG dinucleotides is an important mechanism of variation and regulation in the genome [29-32]. Cytosine methylation, particularly in the promoter region of genes, is often associated with a decrease in transcription [33], and DNA methylation in the first intron and gene expression is correlated and conserved across tissues and vertebrate species [34]. Furthermore, modulation of methylation at CpG sites within the human genome can result in an epigenetic pattern that is specific to individual environmental exposures, and these may contribute to disease [26; 35-37]. For example, environmental factors such as drugs, alcohol, stress, nutrition, bacterial infection, and exercise [36; 38-41] have been associated with methylation changes. A number of these methylation changes have been shown to endure and induce lasting biological changes [36], whereas others are dynamic and transient. For example, alcohol consumption affects genome-wide methylation patterns in a severity-dependent manner [42] and some of these changes revert upon abstinence from alcohol consumption [43]. A similar observation is reported for former tobacco smokers with DNA methylation changes eventually reaching levels close to those who had never smoked tobacco after cessation [44]. Thus, DNA methylation can be indicative of a particular environmental exposure, shed light on the dynamic interaction between the environment and the genome, and provide new insights in to the biological response.

Recreational drug use (an environmental stimuli) has been associated with adverse mental health outcomes particularly in youth [45-49], and epigenetics may play a role in mediating

the biology involved. 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), a longitudinal study of a birth cohort of 1265 children born in 1977 in Christchurch, New Zealand. Users often consume cannabis in combination with tobacco. Unusually, the CHDS cohort contains a subset of cannabis users who have never consumed tobacco, thus enabling an investigation of the specific effects of cannabis consumption, in isolation, on DNA methylation in the human genome.

Methods

Cohort and study design

The Christchurch Health and Development Study includes individuals 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[11-14; 48-54]. A subset of n=96 participants for whom a blood sample was available are included in the current study. Cases (regular cannabis users, n = 48) were matched with controls (n = 48) for sex (n=37 male, n=11 female each group, for additional information see Supplementary 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 [55] diagnostic criteria for cannabis dependence, or had reported using cannabis on a daily basis for a minimum of three years prior to age 28. Cannabis consumption was via smoking, for all participants. The median duration of regular use was 9 years (range 3-14 years). Control participants had never used cannabis or tobacco. Additionally, comprehensive SNP data was available for all participants [56]. 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 [57]). 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 CpGs with adjacent SNVs, or that did not map to a unique location in the genome [58], 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 [59] (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 CpG sites (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 [60]. Linear models were fitted to the methylated/unmethylated or M ratios using limma [61]. 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 [62]. Differentially methylated CpG sites were matched to the nearest neighbouring genes in Hg19 using GRanges [63], and their official gene symbols were tested for enrichment in KEGG 2019 human pathways with EnrichR [64].

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 estimated 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 [65; 66].

Differential methylation

The most differentially methylated CpG sites for cannabis users relative to controls differed in the absence (Table 1) and presence (Table 2) of tobacco smoking. Five individual CpG sites were significantly differentially methylated (P adjusted <0.008) between users and controls when cannabis with tobacco was used (Table 2 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. [44; 67-69]), and cg17739917 is in the same CpG-island as other CpGs

previously shown to be hypomethylated in response to tobacco [70]. Cannabis-only users showed no CpG sites differentially methylated after correction for multiple testing (Table 1 and Figure 2), however the most differentially methylated site was hypermethylation of cg12803068 in the gene *MYO1G*, which has been reported to be hypermethylated in response to tobacco use [67].

To describe the data we chose a nominal P value of 0.001, and observed that both cannabis-only and cannabis with tobacco users showed relatively higher rates of hypermethylation than hypomethylation compared to controls and that the distribution of these CpG sites was similar with respect to annotated genomic features (Table 3). Four CpG sites overlapped between the cannabis-only and cannabis with tobacco users analyses; two were hypermethylated; cg02514528, in the promoter of *MARC2*, and cg27405731 in *CUX1,* and one, cg26542660 in the promoter of *CEP135,* was hypomethylated in comparison to controls. The second most differentially methylated site (ranked by P value) in cannabis-only users was cg02234936 which maps to *ARHGEF1*; this was hypermethylated in the cannabis with tobacco users.

Pathway enrichment analyses

We then took the genes containing differentially methylated CpG sites at P<0.001 for the cannabis-only group that were within genes (that is, not up or downstream in Table 3) 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 adjusted P = 0.03 and enrichment in the glutamatergic synapse and long term potentiation pathway at an adjusted 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 (P=0.05, Figure 4).

Discussion

Many countries have recently adopted, or are considering, lenient polices regarding the personal use of cannabis [71-73]. This approach is supported by the evidence that the prohibition of cannabis can be harmful [53]. Further, the therapeutic benefits of cannabis are gaining traction, most recently as an opioid replacement therapy [74]. However, previous studies, including analyses of the CHDS cohort, have reported an association between cannabis use and poor health outcomes, particularly in youth [75; 76]. Epigenetic mechanisms, including DNA methylation, provide the interface between the environment (e.g. cannabis exposure) and genome. Therefore, we investigated whether changes in an epigenetic mark, DNA methylation, were altered in cannabis users, versus controls, a comparison made possible by the deep phenotyping of the CHDS cohort with respect to cannabis use, and the fact that the widespread practice of mulling or mixing cannabis with tobacco, is not common in New Zealand.

Consistent with previous reports of tobacco exposure, we observed greatest differential methylation in cannabis with tobacco users in the *AHRR* and *F2RL3* genes [44; 67-69]. These changes, however, were not apparent in the cannabis-only data. Only two nominally significantly differentially methylated (P<0.05) CpG sites were observed in both the cannabis-only and cannabis with tobacco analyses. This suggests that tobacco may have a more pronounced effect on DNA methylation and/or dominates any effects of cannabis on the human blood methylome, and that caution should be taken when interpreting similar cannabis exposure studies which do not, or cannot, exclude tobacco smokers. Interestingly, the two nominally significant CpG sites (P<0.05) that overlap between the cannabis-only and the cannabis with tobacco data are located within the *MARC2* and *CUX1* genes, which both have reported roles in brain function; 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 [78].

Cannabis affects the brain, leading to perceptual alterations, euphoria and relaxation [18], and prolonged use is associated with mood disorders, including adult psychosis [7; 8; 49; 79; 80], mania [13], 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 (P<0.05) identified CpG sites within genes involved in brain function and mood disorders, including *MUC3L* [81; 82], *CDC20* [83], *DUS3L* [84], *TMEM190* [85], *FOXB1* [86-88], *KIAA1324L/GRM3* [82; 89-94], *DDX25* [81; 95; 96], *TNRC6B* [97; 98] *and SP9* [99].

Pathway enrichment revealed that hypermethylation in cannabis-only users was over-represented in genes associated with cardiomyopathies and neural signalling. This is consistent with the literature which raises clinical concerns around cardiac complications potentially associated with cannabis use [100-103]. The enrichment of genes associated with neural signalling pathways is also consistent with the literature, including previous analyses of the CHDS cohort, which report associations between cannabis exposure and brain related biology such as mood disorders [7; 12; 48; 49; 51-54; 104; 105]. Our study was limited by sample size, achieving approximately 10% power at $P=10^{-7}$ to detect the largest standardized effect size found. However, while we have not implicated any gene at the genome-wide significance level with respect to differential methylation associated with cannabis-only exposure, our data is strongly suggestive of a role for DNA methylation in the biological response to cannabis, a possibility which definitely warrants further investigations in larger cohorts.

In summary, while tobacco use has declined on the back of state-sponsored cessation programs [106], rates of cannabis use remain high in New Zealand and globally, and might be predicted to increase further with the decriminalisation or legalisation of cannabis use for therapeutic and/or recreational purposes [107]. Therefore, analysis of the potential effects of cannabis (an environmental stimuli) on DNA methylation, an epigenetic mechanism, is timely. Our data is strongly suggestive of a role for DNA methylation in the biological response to cannabis, significantly contributes to the growing literature studying the biological effects of heavy cannabis use, and highlights areas of further analysis in particular with respect to the epigenome.

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References

1. Cressey, D. (2015). The cannabis experiment. Nature 524, 280-283.
2. Goldman, D. (2015). America's cannabis experiment. JAMA Psychiatry 72, 969-970.
3. Stringer, S., Minică, C.C., Verweij, K.J.H., Mbarek, H., Bernard, M., Derringer, J., van Eijk, K.R., Isen, J.D., Loukola, A., Maciejewski, D.F., et al. (2016). Genome-wide association study of lifetime cannabis use based on a large meta-analytic sample of 32 330 subjects from the International Cannabis Consortium. Translational Psychiatry 6, e769.
4. Robson, P. (2001). Therapeutic aspects of cannabis and cannabinoids. The British Journal of Psychiatry 178, 107-115.
5. Amar, M.B. (2006). Cannabinoids in medicine: A review of their therapeutic potential. Journal of ethnopharmacology 105, 1-25.
6. Whiting, P.F., Wolff, R.F., Deshpande, S., Di Nisio, M., Duffy, S., Hernandez, A.V., Keurentjes, J.C., Lang, S., Misso, K., and Ryder, S. (2015). Cannabinoids for medical use: a systematic review and meta-analysis. Jama 313, 2456-2473.
7. Fergusson, D.M., Poulton, R., Smith, P.F., and Boden, J.M. (2006). Cannabis and psychosis. British Medical Journal 332, 172-176.
8. Fergusson, D.M., Hall, W., Boden, J.M., and Horwood, L.J. (2015). Rethinking cigarette smoking, cannabis use, and psychosis. The Lancet Psychiatry 2, 581-582.
9. Radhakrishnan, R., Wilkinson, S.T., and D'Souza, D.C. (2014). Gone to pot–a review of the association between cannabis and psychosis. Frontiers in psychiatry 5, 54.
10. Power, R.A., Verweij, K.J., Zuhair, M., Montgomery, G.W., Henders, A.K., Heath, A.C., Madden, P.A., Medland, S.E., Wray, N.R., and Martin, N.G. (2014). Genetic predisposition to schizophrenia associated with increased use of cannabis. Molecular psychiatry 19, 1201.
11. Gage, S.H., Jones, H.J., Burgess, S., Bowden, J., Smith, G.D., Zammit, S., and Munafò, M.R. (2017). Assessing causality in associations between cannabis use and schizophrenia risk: a two-sample Mendelian randomization study. Psychological medicine 47, 971-980.
12. Horwood, L.J., Fergusson, D.M., Coffey, C., Patton, G., Tait, R., Smart, D., Letcher, P., Silins, E., and Hutchinson, D.M. (2012). Cannabis and depression: An integrative data analysis of four Australasian cohorts. Drug and Alcohol Dependence 126, 369-378.
13. Gibbs, M., Winsper, C., Marwaha, S., Gilbert, E., Broome, M., and Singh, S.P. (2015). Cannabis use and mania symptoms: a systematic review and meta-analysis. Journal of Affective Disorders 171, 39-47.
14. Fergusson, D.M., Boden, J.M., and Horwood, L.J. (2006). Cannabis use and other illicit drug use: Testing the gateway hypothesis. Addiction 101, 556-569.
15. Wang, J.C., Kapoor, M., and Goate, A.M. (2012). The Genetics of Substance Dependence.
16. Adkins, D.E., Clark, S.L., Copeland, W.E., Kennedy, M., Conway, K., Angold, A., Maes, H., Liu, Y., Kumar, G., and Erkanli, A. (2015). Genome-wide meta-analysis of longitudinal alcohol consumption across youth and early adulthood. Twin Research and Human Genetics 18, 335-347.
17. Costello, E.J., Eaves, L., Sullivan, P., Kennedy, M., Conway, K., Adkins, D.E., Angold, A., Clark, S.L., Erkanli, A., McClay, J.L., et al. (2013). Genes, Environments, and Developmental Research: Methods for a Multi-Site Study of Early Substance Abuse. Twin Research and Human Genetics 16, 505-515.
18. Hall, W., and Solowij, N. (1998). Adverse effects of cannabis. The Lancet 352, 1611-1616.
19. Verweij, K.J., Zietsch, B.P., Lynskey, M.T., Medland, S.E., Neale, M.C., Martin, N.G., Boomsma, D.I., and Vink, J.M. (2010). Genetic and environmental influences on cannabis use initiation and problematic use: a meta-analysis of twin studies. Addiction 105, 417-430.
20. Gillespie, N.A., Aggen, S.H., Neale, M.C., Knudsen, G.P., Krueger, R.F., South, S.C., Czajkowski, N., Nesvåg, R., Ystrom, E., and Kendler, K.S. (2018). Associations between personality disorders and cannabis use and cannabis use disorder: a population-based twin study. Addiction.

21. Gillespie, N.A., Neale, M.C., and Kendler, K.S. (2009). Pathways to cannabis abuse: a multi-stage model from cannabis availability, cannabis initiation and progression to abuse. Addiction 104, 430-438.
22. Pasman, J.A., Verweij, K.J., Gerring, Z., Stringer, S., Sanchez-Roige, S., Treur, J.L., Abdellaoui, A., Nivard, M.G., Baselmans, B.M., and Ong, J.-S. (2018). Genome-wide association analysis of lifetime cannabis use (N= 184,765) identifies new risk loci, genetic overlap with mental health, and a causal influence of schizophrenia on cannabis use. bioRxiv, 234294.
23. Visscher, P.M., Brown, M.A., McCarthy, M.I., and Yang, J. (2012). Five years of GWAS discovery. The American Journal of Human Genetics 90, 7-24.
24. Ward, L.D., and Kellis, M. (2012). Interpreting noncoding genetic variation in complex traits and human disease. Nature biotechnology 30, 1095.
25. Fagny, M., Paulson, J.N., Kuijjer, M.L., Sonawane, A.R., Chen, C.-Y., Lopes-Ramos, C.M., Glass, K., Quackenbush, J., and Platig, J. (2017). Exploring regulation in tissues with eQTL networks. Proceedings of the National Academy of Sciences 114, E7841-E7850.
26. Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature genetics 33, 245-254.
27. Petronis, A. (2010). Epigenetics as a unifying principle in the aetiology of complex traits and diseases. Nature 465, 721.
28. Spadafora, R. (2018). The Key Role of Epigenetics in Human Disease. The New England journal of medicine 379, 400.
29. Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.-M., et al. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462, 315-322.
30. Lee, H.J., Hore, T.A., and Reik, W. (2014). Reprogramming the methylome: erasing memory and creating diversity. Cell Stem Cell 14, 710-719.
31. Lowdon, R.F., Jang, H.S., and Wang, T. (2016). Evolution of epigenetic regulation in vertebrate genomes. Trends in Genetics 32, 269-283.
32. Lea, A.J., Vockley, C.M., Johnston, R.A., Del Carpio, C.A., Barreiro, L.B., Reddy, T.E., and Tung, J. (2018). Genome-wide quantification of the effects of DNA methylation on human gene regulation. ELife 7, e37513.
33. Moore, L.D., Le, T., and Fan, G. (2013). DNA methylation and its basic function. Neuropsychopharmacology 38, 23.
34. Anastasiadi, D., Esteve-Codina, A., and Piferrer, F. (2018). Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. Epigenetics & chromatin 11, 37.
35. Jirtle, R.L., and Skinner, M.K. (2007). Environmental epigenomics and disease susceptibility. Nature Reviews Genetics 8, 253-262.
36. Feil, R., and Fraga, M.F. (2012). Epigenetics and the environment: emerging patterns and implications. Nature Reviews Genetics 13, 97-109.
37. Garg, P., Joshi, R.S., Watson, C., and Sharp, A.J. (2018). A survey of inter-individual variation in DNA methylation identifies environmentally responsive co-regulated networks of epigenetic variation in the human genome. PLoS genetics 14, e1007707.
38. Dominguez-Salas, P., Moore, S.E., Baker, M.S., Bergen, A.W., Cox, S.E., Dyer, R.A., Fulford, A.J., Guan, Y., Laritsky, E., and Silver, M.J. (2014). Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. Nature communications 5, 3746.
39. Barres, R., Yan, J., Egan, B., Treebak, J.T., Rasmussen, M., Fritz, T., Caidahl, K., Krook, A., O'Gorman, D.J., and Zierath, J.R. (2012). Acute exercise remodels promoter methylation in human skeletal muscle. Cell metabolism 15, 405-411.
40. Pacis, A., Tailleux, L., Morin, A.M., Lambourne, J., MacIsaac, J.L., Yotova, V., Dumaine, A., Danckaert, A., Luca, F., and Grenier, J.-C. (2015). Bacterial infection remodels the DNA methylation landscape of human dendritic cells. Genome research 25, 1801-1811.

41. Rönn, T., Volkov, P., Davegårdh, C., Dayeh, T., Hall, E., Olsson, A.H., Nilsson, E., Tornberg, Å., Nitert, M.D., and Eriksson, K.-F. (2013). A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. PLoS genetics 9, e1003572.
42. Philibert, R.A., Plume, J.M., Gibbons, F.X., Brody, G.H., and Beach, S.R.H. (2012). The impact of recent alcohol use on genonne wide DNA methylation signatures. Frontiers in Genetics 3, 54.
43. Philibert, R.A., Penaluna, B., White, T., Shires, S., Gunter, T., Liesveld, J., Erwin, C., Hollenbeck, N., and Osborn, T. (2014). A pilot examination of the genome-wide DNA methylation signatures of subjects entering and exiting short-term alcohol dependence treatment programs. Epigenetics 9, 1212-1219.
44. Zeilinger, S., Kühnel, B., Klopp, N., Baurecht, H., Kleinschmidt, A., Gieger, C., Weidinger, S., Lattka, E., Adamski, J., Peters, A., et al. (2013). Tobacco Smoking Leads to Extensive Genome-Wide Changes in DNA Methylation. PLoS ONE 8, e63812.
45. Macleod, J., Oakes, R., Copello, A., Crome, I., Egger, M., Hickman, M., Oppenkowski, T., Stokes-Lampard, H., and Smith, G.D. (2004). Psychological and social sequelae of cannabis and other illicit drug use by young people: a systematic review of longitudinal, general population studies. The Lancet 363, 1579-1588.
46. McGee, R., Williams, S., Poulton, R., and Moffitt, T. (2000). A longitudinal study of cannabis use and mental health from adolescence to early adulthood. Addiction 95, 491-503.
47. Moore, T.H., Zammit, S., Lingford-Hughes, A., Barnes, T.R., Jones, P.B., Burke, M., and Lewis, G. (2007). Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. The Lancet 370, 319-328.
48. Fergusson, D.M., Boden, J.M., and Horwood, L.J. (2015). Psychosocial sequelae of cannabis use and implications for policy: findings from the Christchurch Health and Development Study. Social psychiatry and psychiatric epidemiology 50, 1317-1326.
49. Fergusson, D.M., Horwood, L., and Swain-Campbell, N. (2003). Cannabis dependence and psychotic symptoms in young people. Psychological medicine 33, 15-21.
50. Fergusson, D.M. (2010). Is there a causal linkage between cannabis use and increased risk of psychotic symptoms? (Commentary). Addiction 105, 1336-1337.
51. Fergusson, D.M., Horwood, L.J., and Boden, J.M. (2008). Is driving under the influence of cannabis becoming a greater risk to driver safety than drink driving? Findings from a 25-year longitudinal study. Accident Analysis and Prevention 40, 1345-1350.
52. Fergusson, D.M., Horwood, L.J., and Ridder, E.M. (2005). Tests of causal linkages between cannabis use and psychotic symptoms. Addiction 100, 354-366.
53. Fergusson, D.M., Swain-Campbell, N.R., and Horwood, L.J. (2003). Arrests and convictions for cannabis related offences in a New Zealand birth cohort. Drug and Alcohol Dependence 70, 53-63.
54. Horwood, L.J., Fergusson, D.M., Hayatbakhsh, M.R., Najman, J.M., Coffey, C., Patton, G., Silins, E., and Hutchinson, D.M. (2010). Cannabis use and educational achievement: findings from three Australasian cohort studies. Drug and Alcohol Dependence 110, 247-253.
55. Association, A.P. (1994). Diagnostic and Statistical Manual of Mental Disorders Fourth Edition.(Washington, DC: American Psychiatric Association).
56. Pearson, J.F., Fergusson, D.M., Horwood, L.J., Miller, A.L., Sullivan, P.F., Youfang, L.E., and Kennedy, M.A. (2013). Increased risk of major depression by childhood abuse is not modified by CNR1 genotype. American journal of medical genetics Part B, Neuropsychiatric genetics: the official publication of the International Society of Psychiatric Genetics, 224.
57. Team, R.C. (2019). R: A language and environment for statistical computing. In. (Vienna, Austria.
58. Pidsley, R., Zotenko, E., Peters, T.J., Lawrence, M.G., Risbridger, G.P., Molloy, P., Van Djik, S., Muhlhausler, B., Stirzaker, C., and Clark, S.J. (2016). Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. Genome Biology 17, 208.

59. Triche Jr, T.J., Weisenberger, D.J., Van Den Berg, D., Laird, P.W., and Siegmund, K.D. (2013). Low-level processing of Illumina Infinium DNA methylation beadarrays. Nucleic acids research 41, e90-e90.
60. Jaffe, A.E. (2019). FlowSorted.Blood.450k: Illumina HumanMethylation data on sorted blood cell populations.
61. Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research 43, e47-e47.
62. van Iterson, M., van Zwet, E.W., Heijmans, B.T., and Consortium, B. (2017). Controlling bias and inflation in epigenome-and transcriptome-wide association studies using the empirical null distribution. Genome biology 18, 19.
63. Lawrence, M., Huber, W., Pages, H., Aboyoun, P., Carlson, M., Gentleman, R., Morgan, M.T., and Carey, V.J. (2013). Software for computing and annotating genomic ranges. PLoS computational biology 9, e1003118.
64. Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., and Lachmann, A. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic acids research 44, W90-W97.
65. Ferson, M., Edwards, A., Lind, A., Milton, G., and Hersey, P. (1979). Low natural killer-cell activity and immunoglobulin levels associated with smoking in human subjects. International Journal of Cancer 23, 603-609.
66. Mian, M.F., Lauzon, N.M., Stämpfli, M.R., Mossman, K.L., and Ashkar, A.A. (2008). Impairment of human NK cell cytotoxic activity and cytokine release by cigarette smoke. Journal of leukocyte biology 83, 774-784.
67. Ambatipudi, S., Cuenin, C., Hernandez-Vargas, H., Ghantous, A., Le Calvez-Kelm, F., Kaaks, R., Barrdahl, M., Boeing, H., Aleksandrova, K., and Trichopoulou, A. (2016). Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study. Epigenomics 8, 599-618.
68. Breitling, L.P., Yang, R.X., Korn, B., Burwinkel, B., and Brenner, H. (2011). Tobacco-Smoking-Related Differential DNA Methylation: 27K Discovery and Replication. American Journal of Human Genetics 88, 450-457.
69. Shenker, N.S., Ueland, P.M., Polidoro, S., van Veldhoven, K., Ricceri, F., Brown, R., Flanagan, J.M., and Vineis, P. (2013). DNA methylation as a long-term biomarker of exposure to tobacco smoke. Epidemiology 24, 712-716.
70. Joehanes, R., Just, A.C., Marioni, R.E., Pilling, L.C., Reynolds, L.M., Mandaviya, P.R., Guan, W., Xu, T., Elks, C.E., and Aslibekyan, S. (2016). Epigenetic signatures of cigarette smoking. Circulation: Cardiovascular Genetics 9, 436-447.
71. Kilmer, B. (2017). Recreational cannabis—minimizing the health risks from legalization. New England Journal of Medicine 376, 705-707.
72. Cerdá, M., and Kilmer, B. (2017). Uruguay's middle-ground approach to cannabis legalization. The International journal on drug policy 42, 118.
73. Bifulco, M., and Pisanti, S. (2015). Medicinal use of cannabis in Europe. EMBO reports 16, 130-132.
74. Wiese, B., and Wilson-Poe, A.R. (2018). Emerging evidence for cannabis' role in opioid use disorder. Cannabis and cannabinoid research 3, 179-189.
75. Hall, W. (2015). Challenges in minimizing the adverse effects of cannabis use after legalization. Social Psychiatry and Psychiatric Epidemiology 50, 1013 - 1015.
76. Boden, J.M., and Fergusson, D.M. (2019). Cannabis law and cannabis-related harm. The New Zealand Medical Journal (Online) 132, 7-10.
77. Åberg, K., Adkins, D.E., Bukszár, J., Webb, B.T., Caroff, S.N., Miller, D.D., Sebat, J., Stroup, S., Fanous, A.H., and Vladimirov, V.I. (2010). Genomewide association study of movement-related adverse antipsychotic effects. Biological psychiatry 67, 279-282.

78. Platzer, K., Cogné, B., Hague, J., Marcelis, C.L., Mitter, D., Oberndorff, K., Park, S.M., Ploos van Amstel, H.K., Simonic, I., and van der Smagt, J.J. (2018). Haploinsufficiency of CUX1 Causes Nonsyndromic Global Developmental Delay With Possible Catch-up Development. Annals of neurology 84, 200-207.
79. Caspi, A., Moffitt, T.E., Cannon, M., McClay, J., Murray, R., Harrington, H., Taylor, A., Arseneault, L., Williams, B., and Braithwaite, A. (2005). Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. Biological psychiatry 57, 1117-1127.
80. Henquet, C., Di Forti, M., Morrison, P., Kuepper, R., and Murray, R.M. (2008). Gene-environment interplay between cannabis and psychosis. Schizophrenia bulletin 34, 1111-1121.
81. Consortium, C.-D.G.o.t.P.G. (2013). Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. The Lancet 381, 1371-1379.
82. Consortium, T.A.S.D.W.G.o.T.P.G. (2017). Meta-analysis of GWAS of over 16,000 individuals with autism spectrum disorder highlights a novel locus at 10q24. 32 and a significant overlap with schizophrenia. Molecular autism 8, 1-17.
83. Winham, S.J., Cuellar-Barboza, A., Oliveros, A., McElroy, S., Crow, S., Colby, C., Choi, D.S., Chauhan, M., Frye, M., and Biernacka, J.M. (2014). Genome-wide association study of bipolar disorder accounting for effect of body mass index identifies a new risk allele in TCF7L2. Molecular psychiatry 19, 1010.
84. Linnér, R.K., Biroli, P., Kong, E., Meddens, S.F.W., Wedow, R., Fontana, M.A., Lebreton, M., Tino, S.P., Abdellaoui, A., and Hammerschlag, A.R. (2019). Genome-wide association analyses of risk tolerance and risky behaviors in over 1 million individuals identify hundreds of loci and shared genetic influences. Nature genetics 51, 245.
85. Wong, M.-L., Arcos-Burgos, M., Liu, S., Velez, J.I., Yu, C., Baune, B.T., Jawahar, M.C., Arolt, V., Dannlowski, U., and Chuah, A. (2017). The PHF21B gene is associated with major depression and modulates the stress response. Molecular psychiatry 22, 1015.
86. Davies, G., Lam, M., Harris, S.E., Trampush, J.W., Luciano, M., Hill, W.D., Hagenaars, S.P., Ritchie, S.J., Marioni, R.E., and Fawns-Ritchie, C. (2018). Study of 300,486 individuals identifies 148 independent genetic loci influencing general cognitive function. Nature communications 9, 2098.
87. Cox, A.J., Hugenschmidt, C.E., Raffield, L.M., Langefeld, C.D., Freedman, B.I., Williamson, J.D., Hsu, F.-C., and Bowden, D.W. (2014). Heritability and genetic association analysis of cognition in the Diabetes Heart Study. Neurobiology of aging 35, 1958. e1953-1958. e1912.
88. Lee, J.J., Wedow, R., Okbay, A., Kong, E., Maghzian, O., Zacher, M., Nguyen-Viet, T.A., Bowers, P., Sidorenko, J., and Linnér, R.K. (2018). Gene discovery and polygenic prediction from a genome-wide association study of educational attainment in 1.1 million individuals. Nature genetics 50, 1112.
89. Goes, F.S., McGrath, J., Avramopoulos, D., Wolyniec, P., Pirooznia, M., Ruczinski, I., Nestadt, G., Kenny, E.E., Vacic, V., and Peters, I. (2015). Genome-wide association study of schizophrenia in Ashkenazi Jews. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics 168, 649-659.
90. Ikeda, M., Takahashi, A., Kamatani, Y., Momozawa, Y., Saito, T., Kondo, K., Shimasaki, A., Kawase, K., Sakusabe, T., and Iwayama, Y. (2018). Genome-Wide Association Study Detected Novel Susceptibility Genes for Schizophrenia and Shared Trans-Populations/Diseases Genetic Effect. Schizophrenia bulletin 45, 824-834.
91. Lam, M., Hill, W.D., Trampush, J.W., Yu, J., Knowles, E., Davies, G., Stahl, E., Huckins, L., Liewald, D.C., and Djurovic, S. (2019). Pleiotropic Meta-Analysis of Cognition, Education, and Schizophrenia Differentiates Roles of Early Neurodevelopmental and Adult Synaptic Pathways. bioRxiv, 519967.

92. Li, Z., Chen, J., Yu, H., He, L., Xu, Y., Zhang, D., Yi, Q., Li, C., Li, X., and Shen, J. (2017). Genome-wide association analysis identifies 30 new susceptibility loci for schizophrenia. Nature genetics 49, 1576.
93. Periyasamy, S., John, S., Padmavati, R., Rajendren, P., Thirunavukkarasu, P., Gratten, J., Vinkhuyzen, A., McRae, A., Holliday, E.G., and Nyholt, D.R. (2019). Association of schizophrenia risk with disordered niacin metabolism in an Indian genome-wide association study. JAMA psychiatry 76, 1026-1034.
94. Ripke, S., Neale, B.M., Corvin, A., Walters, J.T., Farh, K.-H., Holmans, P.A., Lee, P., Bulik-Sullivan, B., Collier, D.A., and Huang, H. (2014). Biological insights from 108 schizophrenia-associated genetic loci. Nature 511, 421.
95. Herold, C., Hooli, B.V., Mullin, K., Liu, T., Roehr, J.T., Mattheisen, M., Parrado, A.R., Bertram, L., Lange, C., and Tanzi, R.E. (2016). Family-based association analyses of imputed genotypes reveal genome-wide significant association of Alzheimer's disease with OSBPL6, PTPRG, and PDCL3. Molecular psychiatry 21, 1608.
96. Ripke, S., Sanders, A.R., Kendler, K.S., Levinson, D.F., Sklar, P., Holmans, P.A., Lin, D.-Y., Duan, J., Ophoff, R.A., and Andreassen, O.A. (2011). Genome-wide association study identifies five new schizophrenia loci. Nature genetics 43, 969.
97. Karpova, A., Sanna, P., and Behnisch, T. (2006). Involvement of multiple phosphatidylinositol 3-kinase-dependent pathways in the persistence of late-phase long term potentiation expression. Neuroscience 137, 833-841.
98. Sui, L., Wang, J., and Li, B.-M. (2008). Role of the phosphoinositide 3-kinase-Akt-mammalian target of the rapamycin signaling pathway in long-term potentiation and trace fear conditioning memory in rat medial prefrontal cortex. Learning & memory 15, 762-776.
99. Kichaev, G., Bhatia, G., Loh, P.-R., Gazal, S., Burch, K., Freund, M.K., Schoech, A., Pasaniuc, B., and Price, A.L. (2019). Leveraging polygenic functional enrichment to improve GWAS power. The American Journal of Human Genetics 104, 65-75.
100. Singh, A., Saluja, S., Kumar, A., Agrawal, S., Thind, M., Nanda, S., and Shirani, J. (2018). Cardiovascular complications of marijuana and related substances: a review. Cardiology and therapy 7, 45-59.
101. Rezkalla, S., and Kloner, R.A. (2018). Cardiovascular effects of marijuana. Trends in cardiovascular medicine.
102. Jones, R.T. (2002). Cardiovascular system effects of marijuana. The Journal of Clinical Pharmacology 42, 58S-63S.
103. Goyal, H., Awad, H.H., and Ghali, J.K. (2017). Role of cannabis in cardiovascular disorders. Journal of thoracic disease 9, 2079.
104. Soto, D., Altafaj, X., Sindreu, C., and Bayés, À. (2014). Glutamate receptor mutations in psychiatric and neurodevelopmental disorders. Communicative & integrative biology 7, e27887.
105. Billingsley, K.J., Manca, M., Gianfrancesco, O., Collier, D.A., Sharp, H., Bubb, V.J., and Quinn, J.P. (2018). Regulatory characterisation of the schizophrenia-associated CACNA1C proximal promoter and the potential role for the transcription factor EZH2 in schizophrenia aetiology. Schizophrenia research 199, 168-175.
106. Health, M.o. (2018). Annual Update of Key Results 2017/18: New Zealand Health Survey. In, M.o. Health, ed. (Wellington, Ministry of Health.
107. Organization, W.H. (2016). The health and social effects of nonmedical cannabis use.(World Health Organization).

Table 1 – Top 15 differentially methylated CpG sites in cannabis-only users vs controls. . Beta values with P values, nominal and adjusted by the Benjamini and Hochberg method. Locations are relative to hg19 with gene names for overlapping genes or nearest 5' gene with distance to the 5' end shown.

Table 2 Top 15 differentially methylated CpG sites in cannabis with tobacco users vs controls. Beta values with P values, nominal and adjusted by the Benjamini and Hochberg method. Locations are relative to hg19 with gene names for overlapping genes or nearest 5' gene with distance to the 5' end shown.

Table 3. Summary of CpG sites from cannabis-only and cannabis with tobacco users vs. non-users. Counts of significant sites at P= 0.001 and at a Benjamini and Hochberg adjusted P < 0.05. 'Both' indicates the number of CpG sites of each type that are present and shared across both analyses.

Figure 1 – A Manhattan plot of the genome-wide CpG sites found in the cannabis with tobacco analysis. The Y axis presents -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 the cannabis-only analysis. The Y axis presents -log10(p) values with the most nominally significant different methylated sites labelled with the gene the CpG site resides in.

Figure 3 – Genetic networks enriched within the hypermethylated CpG sites identified in the cannabis-only analysis.

Pathways from KEGG 2019. Genes shown by filled cells are hypermethylated in cannabis-only users and included in named pathway.

Figure 4 – Genetic networks enriched within the hypomethylated CpG sites identified in the cannabis-only users.

Pathways from KEGG 2019. Genes shown by filled cells are hypomethylated in cannabis-only users and included in named pathway.

Table 1

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 2

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 3

	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

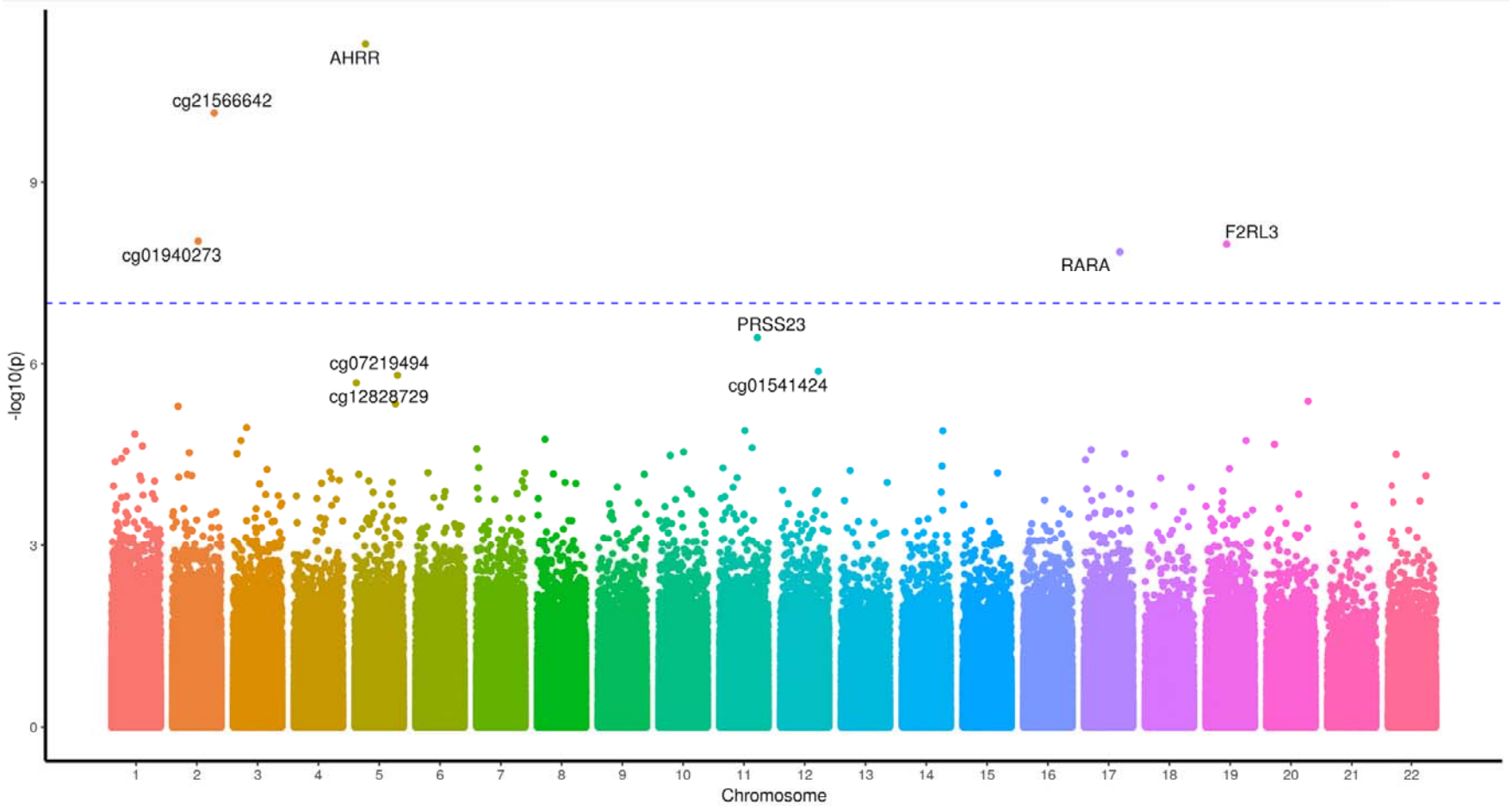

Figure 2

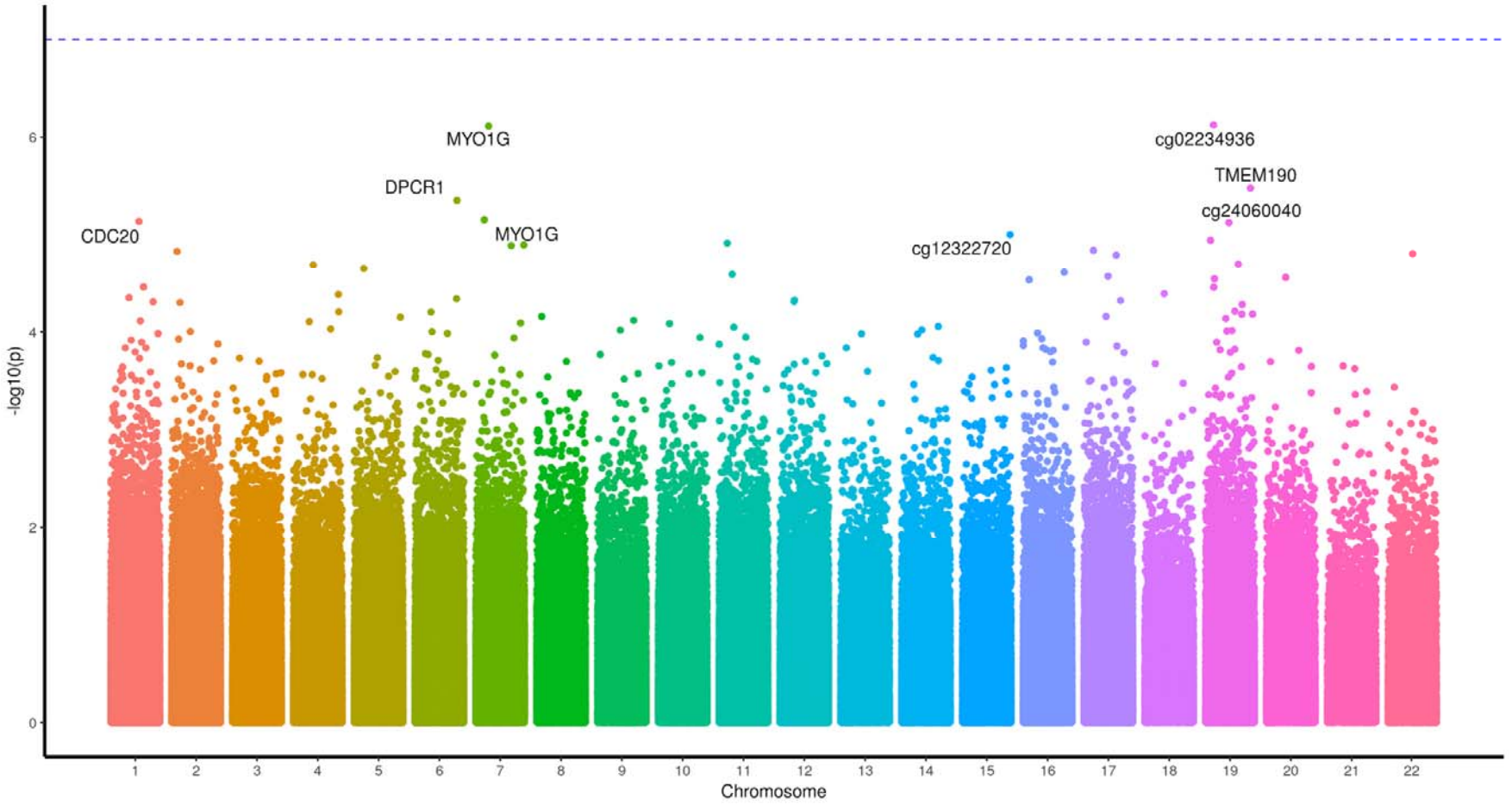

Figure 3

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

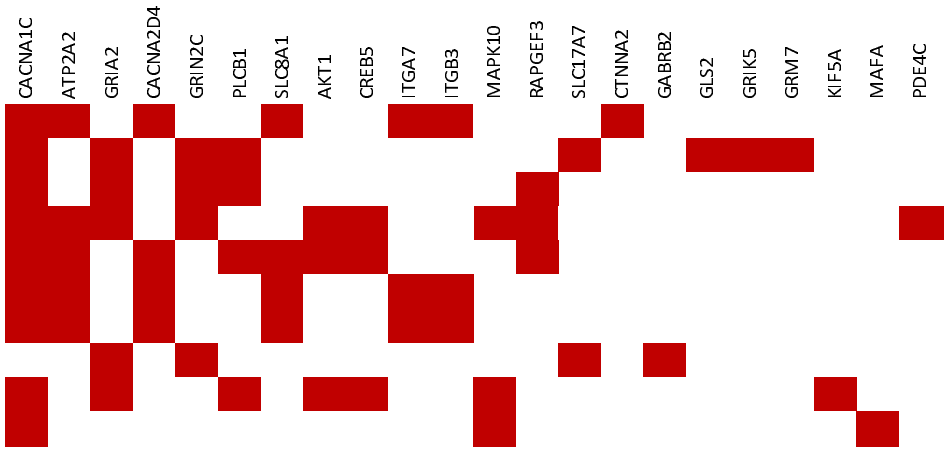

Figure 4.

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