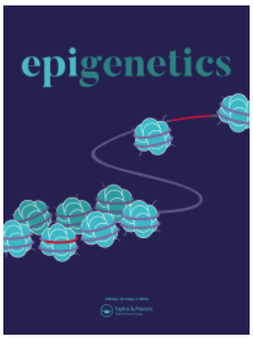

Epigenetics

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/kepi20

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To cite this article: Natàlia Carreras-Gallo, Varun B. Dwaraka, Alejandro Cáceres, Ryan Smith, Tavis L. Mendez, Hannah Went & Juan R Gonzalez (2023) Impact of tobacco, alcohol, and marijuana on genome-wide DNA methylation and its relationship with hypertension, Epigenetics, 18:1, 2214392, DOI: 10.1080/15592294.2023.2214392

To link to this article: https://doi.org/10.1080/15592294.2023.2214392

EPIGENETICS
2023, VOL. 18, NO. 1, 2214392
https://doi.org/10.1080/15592294.2023.2214392

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RESEARCH ARTICLE

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Impact of tobacco, alcohol, and marijuana on genome-wide DNA methylation and its relationship with hypertension

Natàlia Carreras-Gallo [a], Varun B. Dwaraka[b], Alejandro Cáceres [a,c,d], Ryan Smith[b], Tavis L. Mendez[b], Hannah Went[b], and Juan R Gonzalez [a,c,e]

[a]Epidemiology, Barcelona Institute for Global Health (ISGlobal), Barcelona, Spain; [b]TruDiagnostic, Lexington, KY, USA; [c]Centro de Investigación Biomédica en Red en Epidemiología y Salud Pública (CIBERESP), Madrid, Spain; [d]Department of Mathematics, Escola d'Enginyeria de Barcelona Est (EEBE), Universitat Politècnica de Catalunya, Barcelona, Spain; [e]Department of Mathematics, Universitat Autònoma de Barcelona, Barcelona, Spain

ABSTRACT

Tobacco, alcohol, and marijuana consumption is an important public health problem because of their high use worldwide and their association with the risk of mortality and many health conditions, such as hypertension, which is the commonest risk factor for death throughout the world. A likely pathway of action of substance consumption leading to persistent hypertension is DNA methylation. Here, we evaluated the effects of tobacco, alcohol, and marijuana on DNA methylation in the same cohort ($N = 3,424$). Three epigenome-wide association studies (EWAS) were assessed in whole blood using the InfiniumHumanMethylationEPIC BeadChip. We also evaluated the mediation of the top CpG sites in the association between substance consumption and hypertension. Our analyses showed 2,569 CpG sites differentially methylated by alcohol drinking and 528 by tobacco smoking. We did not find significant associations with marijuana consumption after correcting for multiple comparisons. We found 61 genes overlapping between alcohol and tobacco that were enriched in biological processes involved in the nervous and cardiovascular systems. In the mediation analysis, we found 66 CpG sites that significantly mediated the effect of alcohol consumption on hypertension. The top alcohol-related CpG site (cg06690548, P-value = $5.9{\cdot}10^{-83}$) mapped to SLC7A11 strongly mediated 70.5% of the effect of alcohol consumption on hypertension (P-value = 0.006). Our findings suggest that DNA methylation should be considered for new targets in hypertension prevention and management, particularly concerning alcohol consumption. Our data also encourage further research into the use of methylation in blood to study the neurological and cardiovascular effects of substance consumption.

KEY POLICY HIGHLIGHTS

- The consumption of tobacco, alcohol, and marijuana is very high worldwide and is associated with common diseases, like cardiovascular and neurological disorders.
- This study found that tobacco and alcohol have large effects on genome wide DNA methylation while marijuana consumption has nonsignificant effects.
- The genes differentially methylated were enriched in pathways related to neurodevelopment, suggesting the mediation between recreational drug consumption and neurological disorders.
- More remarkably, 66 alcohol related CpG sites significantly mediated the association between heavy drinking and hypertension.
- Our findings suggest that DNA methylation changes should be considered for new targets in disease prevention for recreational drug consumers.

ARTICLE HISTORY
Received 18 October 2022
Revised 13 April 2023
Accepted 9 May 2023

KEYWORDS
Tobacco; Alcohol; Marijuana; DNA methylation; Epigenome-wide association study; Hypertension

Introduction

Tobacco, alcohol, and marijuana are the most commonly used drugs of abuse in the United States [1]. While tobacco and alcohol consumption is legal, marijuana is the most commonly used illicit drug globally [2]. The consumption of these substances is increasing, mainly among adolescents, and the health and social problems associated with them are an important public health concern.

CONTACT Juan R Gonzalez juanr.gonzalez@isglobal.org Barcelona Institute for Global Health (ISGlobal), Barcelona, Spain
Supplemental data for this article can be accessed online at https://doi.org/10.1080/15592294.2023.2214392

Addiction is a major consequence of consuming tobacco, alcohol, and marijuana, which results in the strengthening of all the other health problems associated with them [3].

Cigarette smoking is the leading cause of preventable death and disease in the US and is responsible for approximately 8 million worldwide deaths every year [4]. Most of these deaths arise from cancers (mainly lung cancer), respiratory disease, and cardiovascular disease [5]. Cigarette smoke contains more than 7000 chemical compounds. Among them, 1,3-butadiene is highly associated with cancer risk, acrolein, and acetaldehyde are potential respiratory irritants, cyanide, arsenic, and cresols are the primary sources of cardiovascular risk, and nicotine is the additive component [6]. In addition, nicotine exposure causes well-characterized neurotoxic effects, which are highly important in early development [7].

Light to moderate alcohol intake is associated with reduced risks for total mortality, cardiovascular disease, and diabetes. However, excessive alcohol is the third leading cause of premature death in the US [8]. Heavy alcohol use is associated with a higher risk of cardiovascular disease, diabetes, cirrhosis of the liver, pancreatitis, and cancer [9]. One of the causes of these health consequences is the toxic effect of acetaldehyde, which comes from the metabolization of ethanol [10]. As an example, alcohol and acetaldehyde act as direct toxins to the cardiac myocytes, resulting in contractile dysfunction [11]. Furthermore, excessive exposure to alcohol can lead to severe debilitating diseases of the central and peripheral nervous systems [12].

Among marijuana health impacts, disturbances in the level of consciousness, cognition, perception, affect or behaviour, and other psychophysiological functions and responses are known as short-term effects. Additionally, long-term marijuana consumption can increase the risk of dependence, cognitive impairment, mental disorders (psychoses, depression, anxiety, and suicidal behaviour), and adverse physical health effects such as cardiovascular disease, chronic obstructive pulmonary disease, and cancer [2]. The main components of the cannabis plant are delta-9-tetrahydrocannabinol (THC), which is psychoactive, and cannabidiol (CBD), which is non-psychoactive. These compounds participate in the endocannabinoid system [13].

In summary, the consumption of tobacco, alcohol, and marijuana can lead to similar adverse effects on health, with the neurological and cardiovascular systems being particularly affected. Recent research suggests that epigenetics is a potential mediator between the consumption of toxic substances and the increase in common disease risk [14–17]. DNA methylation, the most studied epigenetic modulation, consists of the addition of a methyl group (–CH3) in the cytosine nucleotide without changing the DNA sequence. It occurs in the context of CpG sites, which are defined as adjacent cytosine and guanine nucleotides by a phosphate group. DNA methylation is dynamic and can be modified by genetic factors, disease, environmental exposures, and lifestyle [17–19]. Moreover, DNA methylation can change during the lifetime and across tissues and cell types [20,21].

Although genetic mechanisms have been the focus of understanding human diseases, the disruption of the epigenetic balance can result in the modulation of gene expression. Consequently, epigenetic disruption can cause several major pathologies, including cancer and cardiovascular disease [14]. Tobacco smoking is one of the exposures with a higher impact on the DNA methylation of smokers [22–25]. Its effect has also been observed in the newborns' cord blood of mothers who smoked during pregnancy [26–28]. Many studies also demonstrate that alcohol consumption produces methylation changes at the CpG site level [29–34]. Conversely, only a few studies have demonstrated the effects of marijuana consumption on DNA methylation, all of which have shown small effects [35,36].

Hypertension, also known as high blood pressure, is a medical condition in which the blood pressure in the arteries is persistently elevated. It affects one billion people and is the most common risk factor for death worldwide [37]. There are many factors associated with a higher risk of hypertension, including body mass index, tobacco use, physical activity, and alcohol consumption, among others [38]. Light to moderate alcohol

consumption seems to protect against hypertension because it decreases systolic and diastolic blood pressure. However, excessive intake accounts for about 16% of cases of hypertension worldwide [39,40]. Cigarette smoking enhances hypertension by inducing cardiovascular mitochondrial oxidative stress [41,42]. On the other hand, some studies evaluating the effect of marijuana consumption on blood pressure have revealed different results. For instance, Abuhasira *et al*. demonstrated the therapeutic effect of marijuana in reducing blood pressure in hypertensive patients [43]. However, other studies revealed an increase in blood pressure after marijuana consumption [44,45]. In light of the above, we hypothesized that changes in DNA methylation produced by substance consumption may partially explain its relationship with hypertension.

To this end, in this study we aimed to: i) perform a genome-wide association study of DNA methylation with tobacco, alcohol, and marijuana consumption; ii) identify the physiological pathways whose methylation is affected by those recreational drugs; iii) evaluate the mediation between substance consumption and hypertension by methylation changes at the CpG site level.

Materials and methods

The study cohort

Our study sample included 3,590 individuals from the TruDiagnostic DNA biobank recruited between October 2020 and February 2022. Those individuals have taken the commercial TruDiagnostic TruAge test and methylation data was generated from them. This is an EEUU population-based cohort aged between 13 and 97 years old. Among them, 58.7% are male. Demographic and substance use characteristics of the samples that met the QC requirements (N = 3,424) are displayed in Table 1. As this testing is priced to the consumer at approximately $500, this study cohort is relatively more affluent than random sampling or traditional banked cohorts. Additionally, these individuals may experience a healthy donor effect whereby they seek preventative medicine and have fewer comorbidities than normal patient populations, as is the case with blood donors [46,47]. The study involving human participants was reviewed and approved by the IRCM IRB. The patients/participants provided their written informed consent to participate in this study.

DNA methylation Assessment

Peripheral whole blood was collected by the lancet and capillary method into lysis buffer and DNA extract, and 500 ng of DNA of bisulphite were converted using the EZ DNA Methylation kit (Zymo Research) according to the manufacturer's instructions. Bisulphite-converted DNA samples were randomly assigned to a chip well on the Infinium HumanMethylationEPIC BeadChip, amplified, hybridized onto the array, stained, washed, and imaged with the Illumina iScan SQ instrument to obtain raw image intensities.

Meffil R package [48] was used for the preprocessing of DNA methylation data. In the sample quality control, we removed the sex detection mismatches and the sex detection outliers (based on the difference between median chromosome Y and chromosome X probe intensities). We also discarded those samples whose predicted median methylated signal was more than 3 standard deviations from the expected. We excluded the outliers based on deviations from mean values for control probes (dye bias, bisulphite 1, and bisulphite 2). Finally, we removed those samples with more than 5% of undetected probes (detection *P-value* larger than 0.01) or with a low number of beads (less than 3). This quality control resulted in 3,424 individuals, indicating that 90,3% of the samples met our QC standards. In the feature quality control, we removed those probes undetected or with low bead numbers in more than 5% of the samples. We used InfiniumAnnotation [49] to filter probes where the 30bp 3'-subsequence of the probe is non-unique, probes with INDELs, probes with extension base inconsistent with specified colour channel (type-I) or CpG (type-II) based on mapping, probes with a SNP in the extension base that causes a colour channel switch from the official annotation, and probes where 5bp 3'-subsequence overlap with any of the SNPs with global population frequency higher than 1%. The functional normalization method was further applied based on the first 10 principal components of the control probes. Consequently, the number of CpG probes kept was 745,150, which represents 86% of the total EPIC array manifest. CpG sites

Table 1. Characteristics of participants in the TruDiagnostic DNA Biobank.

	$N = 3424$
Sex, male	2010 (58.7%)
Age in years, mean (range)	52.9 (13.3–97.8)
Ethnicity	
European	2584 (75.5%)
African American or Black	70 (2.0%)
Asian	41 (1.2%)
Latino or Hispanic	276 (8.1%)
Middle Eastern or North African	76 (2.2%)
Native American or Alaska Native	26 (0.8%)
Pacific Islander or Oceanian	23 (0.7%)
Sub-Saharan African	7 (0.2%)
Other	321 (9.4%)
BMI (kg/m^2), median (range)	25.4 (10.1–58.2)
Tobacco consumption	
None	3275 (95.6%)
Less than 1 cigarette per week	48 (1.4%)
Less than 1 cigarette per day	25 (0.7%)
1–5 cigarettes per day	27 (0.8%)
6–10 cigarettes per day	21 (0.6%)
11–20 cigarettes per day	20 (0.6%)
More than 20 cigarettes per day	8 (0.2%)
Alcohol consumption	
Never	634 (18.5%)
On special occasions	976 (28.5%)
Once per week	578 (16.9%)
3–5 times per week	794 (23.2%)
Regularly	442 (12.9%)
Marijuana consumption	
Missing	149
Never	2908 (88.8%)
On special occasions	180 (5.5%)
Once per week	46 (1.4%)
3–5 times per week	73 (2.2%)
Regularly	68 (2.1%)

Note: All the continuous variables are shown as mean (range) and the categorical variables as n (%).
BMI: body mass index.

were annotated to genes using EPIC Illumina annotation ilm10b4.hg19. Blood cell type proportions were estimated using the blood gse35069 complete cell type methylation profile references from the *meffil* package. We then performed a surrogate variable analysis (SVA) to remove the batch effects using the *SmartSVA* package [50]. We estimated the number of surrogate variables (SVs) using the *isva* package [51]. Methylation levels were expressed as residual values after adjusting beta values for the first 60 SVs.

Exposure and clinical history assessment

During the recruitment of participants, they were asked to complete a survey that included questions about personal information, medical history, social history, lifestyle, and family history. Alcohol and marijuana consumption was assessed on a 5-point scale ('never' to 'regularly'). Participants also reported their level of smoking according to 7 possible answers ('none' to 'more than 20 cigarettes per day'). Regarding the medical history, the survey covered information about the blood type, medications and supplements, and diagnosis of any type of disease (cardiovascular, respiratory, skin and hair, endocrine, gastrointestinal, genitourinary, musculoskeletal, neuropsychological, reproductive, immune, and cancer). The main clinical outcome of this study was hypertension, assessed as a dichotomic variable (affected and unaffected.

Statistical analyses and reproducibility

Epigenome-wide association analysis

The epigenome-wide association study (EWAS) was performed using the *MEAL* Bioconductor package [52]. We performed a differential mean analysis on different substance consumption (tobacco, alcohol, and marijuana) using the

function *runPipeline* that calls *limma* [53]. Based on previous analyses [32], we adjusted all the regression models by sex, age, ethnicity, body mass index (BMI), level of education, alcohol and tobacco consumption (except when they were the variable of interest), slide, cell type, and surrogate variables. For each substance, we fitted models

$$E_{j=}\alpha_j+\beta_j S+\Sigma_r\gamma_r C_r+\varepsilon_j \quad (1)$$

where E_j denotes the methylation level vector across individuals at probe j (j = 1, … 745150), S is the individuals' consumption (separated models for alcohol, smoking, and marijuana where fitted) with its associated effect, β_j, C_r is the r adjusting covariate and its effect γ_r, and ε_j is the noise that follows the distribution of methylation levels with mean 0. Adjusted *P-values* were calculated using Bonferroni's correction for considering multiple comparisons. The inflation or deflation of *P-values* across the methylome was assessed with Q-Q plots and lambda values [54].

Enrichment analysis

We performed an enrichment in Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathways [55,56] using the *enrichKEGG* and *enrichGO* functions from the *clusterProfiler* Bioconductor package [57], respectively. Using the same package, we also evaluated the over-representation of diseases using the DisGeNET platform which contains 1,134,942 gene-disease associations [58]. Associations were corrected for multiple comparisons using Bonferroni adjustment, as computed by *clusterProfiler*.

Comparison of the results with previous studies

For the validation of our results, we used publicly available data from the EWAS catalogue. This catalogue includes published epigenome-wide association studies from PubMed using the search terms 'epigenome-wide' OR 'epigenome wide' OR 'EWAS' OR 'genome-wide AND methylation' OR 'genome wide AND methylation.' Studies are selected according to the year of publication (>2010), the use of human samples, and the number of genome-wide CpG sites (>100,000) and individuals (>100). Reported CpG sites have nominal *P*-values lower than $1{\cdot}10^{-4}$. The data for the EWAS Catalogue is then manually curated. We obtained separate datasets for each drug of abuse containing information on the associations between DNA methylation and tobacco, alcohol, and marijuana consumption. The dataset for tobacco smoking included 30 publications, while the dataset for alcohol consumption had 6 publications, and the dataset for marijuana consumption contained 2 publications. After excluding associations that lacked information on the beta value, we obtained 26 publications for smoking, no publications for alcohol consumption, and 1 publication for marijuana consumption. For CpG sites that were reported in more than one study, we performed a metanalysis using the *meta* package [59]. However, due to the high heterogeneity between studies, we reported the proportion of studies that showed the same direction of effect as our study.

Estimation of epigenetic clocks

We further evaluated whether the three behaviours had an impact on epigenetic age. To calculate the principal component-based epigenetic clock for the Horvath multi-tissue clock, Hannum clock, PhenoAge clock, GrimAge clock, and telomere length we used the custom R script available via GitHub (https://github.com/MorganLevineLab/PC-Clocks). Non-principal component-based (non-PC) Horvath and Hannum epigenetic estimates were calculated using the *agep* function available in the *wateRmelon* R package [60], and non-PC PhenoAge was calculated using the *methyAge* function in the *ENMix* R package [61]. Finally, the pace of ageing clock, DunedinPACE, was calculated using the *PACEProjector* package available via GitHub (https://github.com/danbelsky/DunedinPACE).

Mediation analysis between recreational drug use and hypertension

To investigate the relationship between tobacco, alcohol, and marijuana consumption and hypertension, we conducted logistic regression analyses. Next, we performed a mediation analysis to explore whether methylation played a role in the relationship between recreational drug use and hypertension. We first identified CpG sites that were significantly associated with those behaviours

and hypertension after correcting for multiple comparisons using Bonferroni. Then, we conducted a univariate mediation analysis for each significant CpG site using the *mediate* function from the *mediation* package [62]. This analysis allowed us to estimate the total effect, the effect of substance consumption on CpG methylation, and the effect of the mediator and substance consumption on hypertension. Finally, we performed a causal mediation analysis and estimated the average causal mediation effects, average direct effects, total effect of the independent variable on the dependent variable, and the proportion of the effect of the independent variable on the dependent variable that goes through the mediator. We adjusted all models for the same covariates as in the EWAS.

Results

We analysed 3,424 individuals from the TruDiagnostic DNA Biobank recruited from the general population in EEUU. Table 1 presents demographic and substance use characteristics. The mean age was 52.9 years (range: 13.3–97.8) and 58.7% were male. The participants were classified according to 7 ethnic groups and 'other' for those who had a mixed ethnicity. Most participants were Europeans (75.5%), and Latino American was the second most common ethnicity (8.1%). There were 149 current tobacco smokers, classified into seven groups according to the number of cigarettes smoked, and 3,275 non-smokers. Regarding alcohol consumption, there were 2,790 drinkers grouped by consumption frequency and 634 non-drinkers. Marijuana consumption was also classified according to consumption frequency. In total, 2,908 did not smoke marijuana, and 367 smoke marijuana at least on special occasions. It is worth noting that out of the participants, 465 individuals reported consuming more than one drug, at least on special occasions. On the other hand, 584 participants reported not consuming any of the drugs included in this study. Among those who reported using recreational drugs, the largest overlap was observed between alcohol and marijuana, with 299 individuals indicating consumption of both substances. Notably, 98 participants reported consuming both tobacco and alcohol, highlighting the co-occurring use of different substances (Additional File 2: Fig. S1).

Genome-wide effect of tobacco smoking on DNA methylation

We tested the association between the level of smoking (codified as a 7-point scale from 'none' to 'more than 20 cigarettes per day;' see Table 1) with each CpG site using linear regression models run in the *MEAL* R package [52]. We found 528 CpG sites associated with smoking levels after correcting for multiple comparisons and genomic inflation was not observed ($\lambda = 1.031$). Table 2 lists the top 15 CpG sites for tobacco smoking (see Additional File 1: Table S1 for all the significant CpG sites). Figure 1a shows how the CpG sites are distributed in the genome using a Manhattan plot. Among tobacco-related methylation sites, 68.2% were hypomethylated (that is, lower DNA methylation associated with higher tobacco consumption). From the 528 probes differentially methylated, 374 CpG sites were mapped to 344 unique genes. *AHRR, GFI1, PRSS23*, and *IMMP2L* had 10, 6, 4, and 4 probes differentially methylated, respectively. Moreover, these 344 genes were enriched in morphine addiction (P-value $= 4.3 \cdot 10^{-6}$), dopaminergic synapse (P-value $= 1.1 \cdot 10^{-4}$), and cholinergic synapse (P-value $= 1.7 \cdot 10^{-4}$) KEGG pathways (Additional File 2: Fig. S2). Consistent with previous studies, cg05575921 was the top-ranked CpG with a $P\text{-value} = 1.3 \cdot 10^{-226}$. We further demonstrated that the effect of tobacco in this CpG site was proportional to the number of cigarettes smoked (Figure 2a). In addition, we cross-referenced our findings with those previously documented in the EWAS catalogue. Out of the 528 CpG sites that displayed differential methylation in our investigation, 183 had already been documented in the EWAS catalogue, with 181

Table 2. Top 15 differentially methylated CpG sites by tobacco consumption.

CpG	chr	position	Gene Symbol	Gene Group	Less than 1 per week	Less than 1 per day	1–5 per day	6–10 per day	11–20 per day	More than 20 per day	F	P-Value	Adjusted P-Value	EWAS catalogue (same direction/total studies)
cg05575921	chr5	373378	*AHRR*	Body	−0.028	−0.054	−0.097	−0.216	−0.183	−0.217	208.2	1.3E−226	9.7E−221	26/26
cg21566642	chr2	233284661			−0.030	−0.058	−0.066	−0.109	−0.111	−0.160	59.7	1.6E−70	5.9E−65	22/22
cg01940273	chr2	233284934			−0.016	−0.034	−0.045	−0.066	−0.069	−0.109	53.6	2.4E−63	5.9E−58	22/22
cg03636183	chr19	17000585	*F2RL3*	Body	−0.007	−0.017	−0.029	−0.064	−0.053	−0.094	50.9	2.8E−60	5.2E−55	21/21
ch.6.2768623F	chr6	144037285	*PHACTR2*	Body	−0.002	−0.001	0.000	0.009	−0.005	0.350	49.1	4.7E−58	7.0E−53	Not reported
cg21161138	chr5	399360	*AHRR*	Body	−0.008	−0.016	−0.015	−0.071	−0.043	−0.069	40.6	5.7E−48	7.1E−43	16/16
cg17739917	chr17	38477572	*RARA*	5′UTR	−0.008	−0.053	−0.034	−0.069	−0.082	−0.116	38.2	4.5E−45	4.8E−40	7/7
cg15928392	chr2	81694446			0.000	0.000	0.001	−0.001	−0.002	−0.106	34.1	3.4E−40	3.2E−35	Not reported
cg16774290	chr7	4824628	*AP5Z1*	Body	0.000	0.000	0.000	−0.001	0.000	−0.100	33.2	4.1E−39	3.4E−34	Not reported
cg11173636	chr10	65632259			−0.008	0.017	0.024	0.018	0.339	−0.020	30.5	8.5E−36	6.3E−31	Not reported
cg09935388	chr1	92947588	*GFI1*	Body	−0.018	−0.030	−0.029	−0.100	−0.069	−0.134	30.0	3.5E−35	2.4E−30	20/20
cg26703534	chr5	377358	*AHRR*	Body	−0.004	−0.013	−0.016	−0.052	−0.034	−0.049	29.9	4.0E−35	2.4E−30	14/14
cg14990808	chr6	28493651	*GPX5*	TSS200	−0.002	−0.022	0.005	0.002	0.001	0.246	26.3	1.1E−30	6.2E−26	Not reported
cg25648203	chr5	395444	*AHRR*	Body	−0.006	−0.005	−0.011	−0.047	−0.037	−0.057	24.8	6.2E−29	3.3E−24	18/18
cg04176674	chr14	21121564			−0.004	−0.002	0.001	0.005	0.000	0.343	24.7	7.8E−29	3.9E−24	Not reported

Note: The CpG sites are annotated based on the chromosome (chr), the position (pos), the gene symbol from HGNC, and the gene group (based on the position of the CpG regarding the nearest gene). Each CpG site has a beta value for each consumer group vs non-consumers, a F-statistic (F), a nominal *p-value*, and an adjusted *p-value* by Bonferroni. The last column represents the number of studies in the EWAS catalogue that reported the same effect direction compared to the total studies that reported the beta value for the specific CpG site.

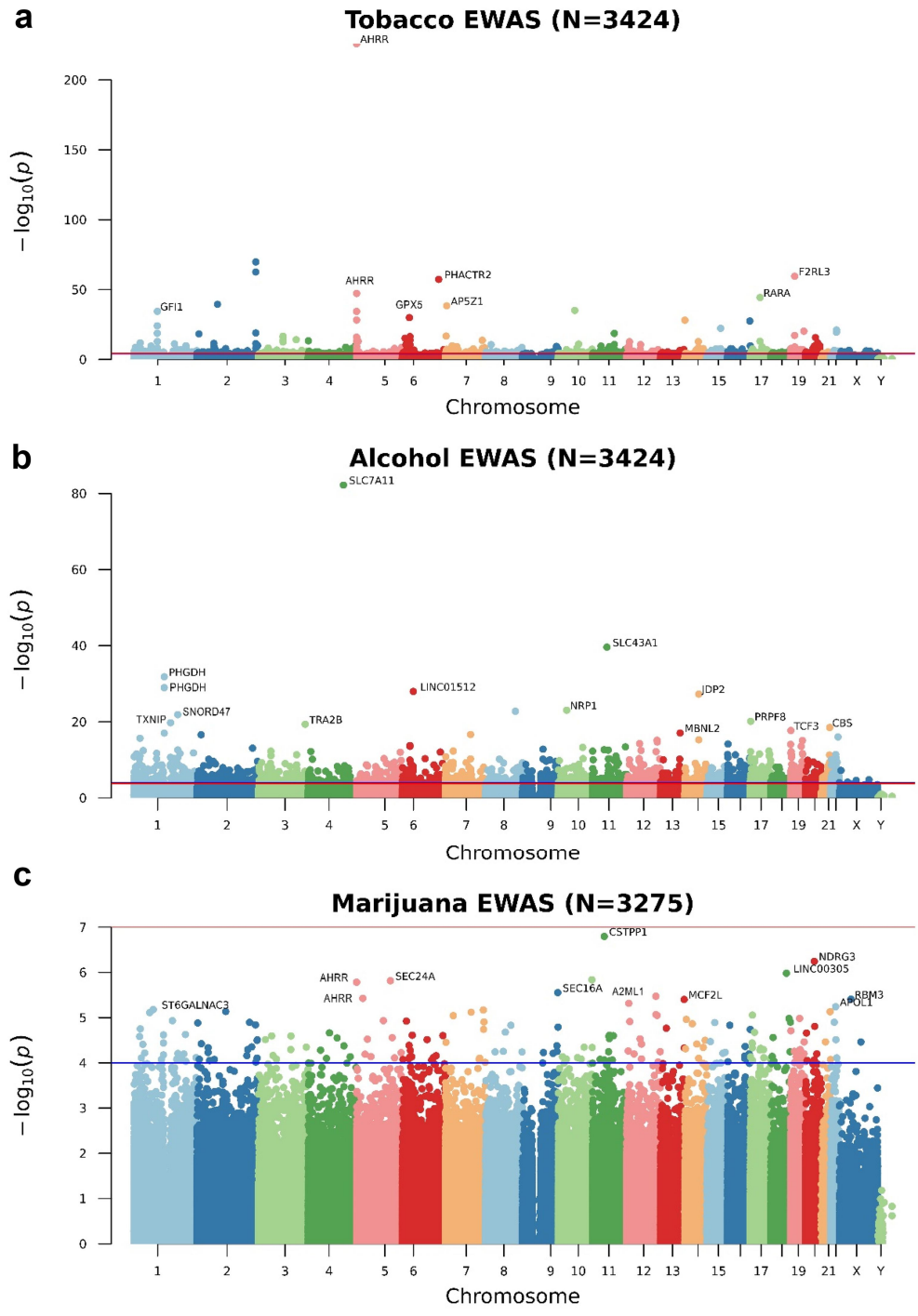

Figure 1. Manhattan plots of the epigenome-wide association study (EWAS) of tobacco (a), alcohol (b), and marijuana (c) consumption. The Y-axis represents the -log10(p) values and the X-axis the position of the CpG sites within the chromosome. The blue line is the suggestive nominal p-value threshold (0.0001) and the red line is the p-value adjusted threshold lower than 0.05.

showing the same direction of change and 2 exhibiting an opposing direction. Among the 345 not-reported, 6 of them were in the top 15 CpG sites in our data, evidencing that tobacco may have important effects on them (Table 1).

Genome-wide effect of alcohol consumption on DNA methylation

We identified 2,569 CpG sites differentially methylated according to alcohol consumption frequency (5 levels from 'never' to 'regularly;' see Table 1). Model

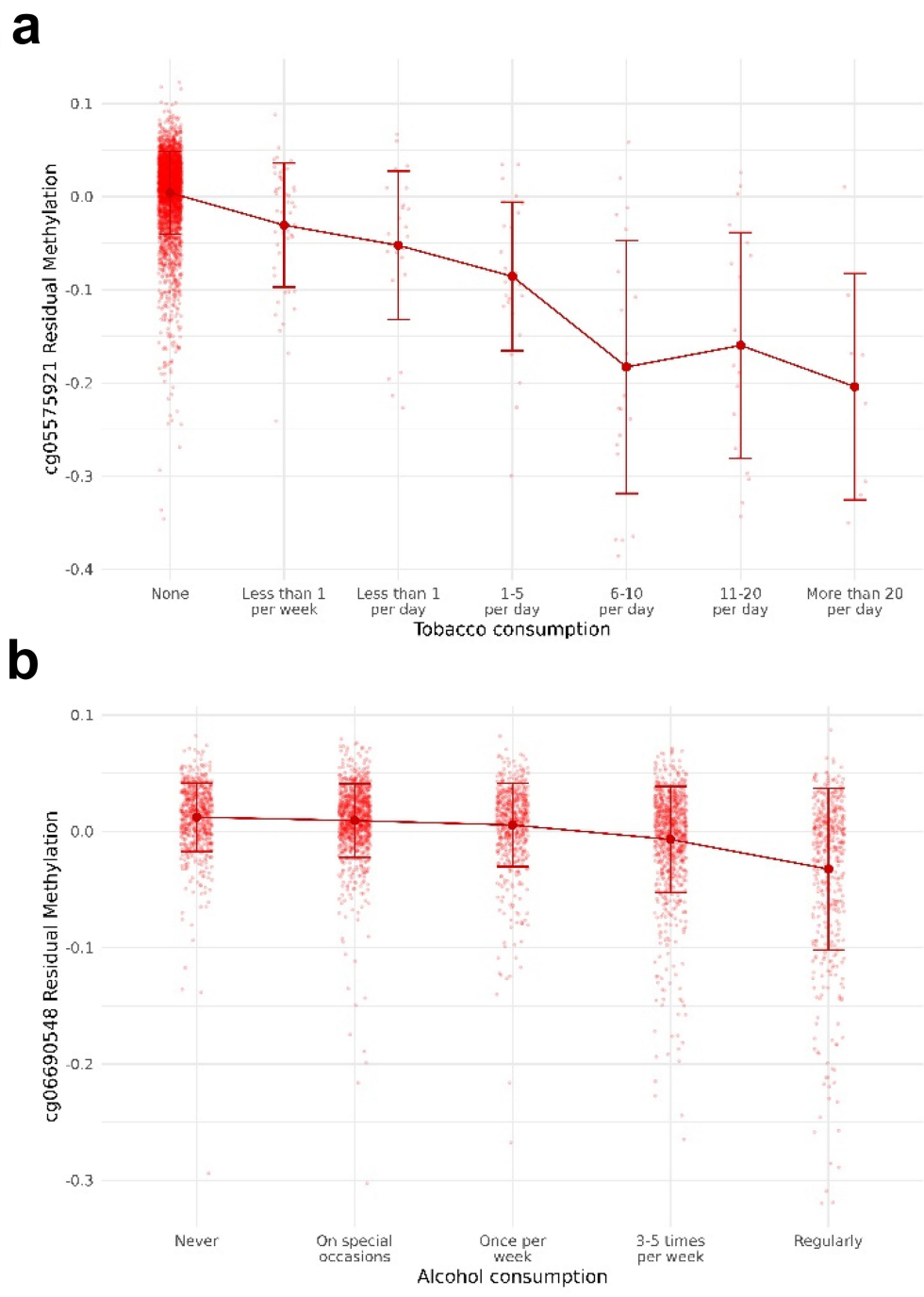

Figure 2. Boxplots showing the association between CpG methylation and substance consumption. (a) Association between cg05575921 methylation (AHRR) and tobacco consumption. (b) Association between cg06690548 methylation (SLC7A11) and alcohol consumption. The Y-axis represents the residuals for beta values after adjusting by covariates. The X-axis represents the number of cigarettes smoked and the frequency of drinking, respectively. Methylation means for each tobacco consumption level are represented with their 95% confidence intervals.

fitting showed no indication of genomic inflation ($\lambda = 1.044$). The top 15 CpG sites are shown in Table 3 and all the epigenome-wide significant CpG sites are listed in Additional File 1: Table S3 and represented as a Manhattan Plot in Figure 1b. Among them, 36.9% were hypomethylated for regular consumers compared with non-consumers. However, the percentage of hypomethylated probes was increased for the most significant probes. Among the 2,569 alcohol-related methylation sites, 609 were intergenic

Table 3. Top 15 differentially methylated CpG sites by alcohol consumption.

CpG	chr	position	Gene Symbol	Gene Group	On special occasions	Once per week	3–5 times per week	Regularly	F	P-Value	Adjusted P-Value	Lohof et al (direction)
cg06690548	chr4	139162808	*SLC7A11*	Body	−0.003	−0.005	−0.015	−0.031	103.1	5.9E−83	4.4E−77	–
cg11376147	chr11	57261198	*SLC43A1*	Body	−0.001	−0.002	−0.006	−0.010	49.3	2.4E−40	8.9E−35	–
cg26457483	chr1	120256112	*PHGDH*	Body	−0.001	−0.004	−0.017	−0.028	39.7	1.5E−32	3.8E−27	–
cg14476101	chr1	120255992	*PHGDH*	Body	−0.002	−0.005	−0.016	−0.031	36.2	1.1E−29	2.0E−24	–
cg18120259	chr6	43894639	*LINC01512*	Body	−0.002	−0.004	−0.009	−0.019	35.0	1.1E−28	1.6E−23	–
cg06088069	chr14	75895604	*JDP2*	5′UTR	−0.002	−0.003	−0.007	−0.013	34.2	5.0E−28	6.1E−23	–
cg21998542	chr10	33605101	*NRP1*	Body	−0.002	−0.005	−0.009	−0.019	29.0	9.2E−24	9.8E−19	–
cg15837522	chr8	117892654			−0.003	−0.011	−0.022	−0.031	28.7	1.7E−23	1.6E−18	–
cg06644515	chr1	173834831	*SNORD47; GAS5; SNORD81; SNORD80; SNORD78; SNORD79*	TSS1500; Body; TSS1500; TSS1500; TSS200; TSS1500	−0.001	−0.001	−0.007	−0.013	27.6	1.3E−22	1.1E−17	–
cg12116137	chr17	1576449	*PRPF8*	Body	0.001	0.001	0.008	0.018	25.5	7.5E−21	5.5E−16	++
cg19693031	chr1	145441552	*TXNIP*	3′UTR	−0.004	−0.003	−0.011	−0.026	25.1	1.8E−20	1.2E−15	–
cg12825509	chr3	185648568	*TRA2B*	Body	0.000	−0.004	−0.010	−0.016	24.6	4.5E−20	2.8E−15	–
cg14346162	chr21	44490229	*CBS*	Body	0.001	0.004	0.009	0.014	23.5	3.1E−19	1.8E−14	Not reported
cg12973487	chr19	1623075	*TCF3*	Body	0.004	0.005	0.007	0.014	22.6	2.0E−18	1.0E−13	++
cg05713943	chr13	97912352	*MBNL2*	5′UTR	0.001	0.000	−0.005	−0.008	21.8	8.7E−18	4.2E−13	–

Note: The CpG sites are annotated based on the chromosome (chr), the position (pos), the gene symbol from HGNC, and the gene group (based on the position of the CpG regarding the nearest gene). Each CpG site has a beta value for each consumer group vs non-consumers, a F-statistic (F), a nominal *p-value*, and an adjusted *p-value* by Bonferroni. We also included one column with the direction of the effect in a previous study (Lohoff et al (31)) for those that were significant in their analysis.

and 1,960 were annotated to 1,670 unique genes. Five genes had seven or more significant probes mapping to their locus, including *RPTOR* (11 probes), *JARID2* [8], and *ABCG1* [8]. The enrichment revealed an over-representation of autistic disorder (P-value = $4.2 \cdot 10^{-7}$), acquired scoliosis and curvature of the spine (P-value = $2.7 \cdot 10^{-4}$), small nose (P-value = $2.5 \cdot 10^{-4}$), small midface (P-value = $6.1 \cdot 10^{-5}$), and self-injurious behaviour (P-value = $1.5 \cdot 10^{-4}$) (Additional File 2: Fig. S3A). These genes were also enriched in the PI3k-Akt signalling pathway (P-value = $4.0 \cdot 10^{-4}$), which is involved in the cell cycle, cholinergic synapse (P-value = $2.7 \cdot 10^{-5}$), and longevity regulating pathway (P-value = $6.7 \cdot 10^{-5}$) (Additional File 2: Fig. S3B). The GO enrichment revealed regulation of Wnt signalling pathway (P-value = $3.2 \cdot 10^{-8}$) and heart growth (P-value = $2.6 \cdot 10^{-5}$) (Additional File 2: Fig. S3C). The top CpG site (cg06690548, *P-value* = $5.9 \cdot 10^{-83}$) mapped to the *SLC7A11* gene and its methylation was significantly reduced proportionally to the alcohol consumption (Figure 2b). Due to the absence of beta value information in the studies included in the EWAS catalogue, we compared our results with the largest single-cohort EWAS of alcohol consumption performed by Lohoff et al [31]. In this study, they found 2,504 CpG sites and 909 were overlapping with our study. All the CpG sites overlapping had the same direction of the effect.

Genome-wide effect of marijuana consumption on DNA methylation

In the EWAS for the frequency of marijuana use (codified as a 5-point scale from 'never' to 'regularly;' see Table 1), we did not find any CpG site with a *P-value* adjusted lower than 0.05 (Figure 1c). However, we used the *P*-value threshold from the EWAS catalogue (*P-value* < $1 \cdot 10^{-4}$) and we found 195 CpG sites at a suggestive significant level (Additional File 1: Table S3). From them, almost 50%were hypomethylated for regular consumption compared to no consumption (Table 4). Gene symbols for the 195 CpG sites were tested for enrichment in

KEGG pathways and Gene Ontology (GO) and we found an enrichment of myelin assembly (P-value = $8.0{\cdot}10^{-6}$). We did not find CpG sites overlapping between our results and the ones reported in the EWAS catalogue. This may be in part due to the differences in the variable of interest and the study population, such as the evaluation of the effect of cannabis use on non-Hispanic white women and the risk of breast cancer [36].

Comparison between recreational drugs

We compared the differentially methylated CpG sites for the different drugs of abuse. Since no CpG sites were significant for marijuana consumption after correcting by multiple comparisons, we focused on comparing the results between tobacco and alcohol. We found that 12 CpG sites were overlapping between tobacco and alcohol, and nine of them had the same direction effect in both analyses (Figure 3a). Next, we extracted the genes where all the differentially methylated CpG sites were annotated and compared the alcohol and tobacco related genes. We observed a larger overlap and found that 61 genes were differentially methylated by both substances (Figure 3b). To determine whether these genes were involved in similar pathways, we performed an enrichment analysis, and the results revealed that these genes were implicated in various biological processes, including positive regulation of heart rate (P-value = $5.3{\cdot}10^{-5}$), inositol lipid-mediated signal (P-value = $1.7{\cdot}10^{-4}$), and positive regulation of blood circulation (P-value = $1.9{\cdot}10^{-4}$), among others (Additional File 2: Fig. S4).

Then, we evaluated whether recreational drug consumption was associated with multiple epigenetic clocks. For tobacco smoking, we found a significant association with telomere length (β = −0.01, P-value = $1.2{\cdot}10^{-3}$), Grim Age Principal Component (β = 0.72, P-value = $1.6{\cdot}10^{-17}$), and DunedinPACE (β = 0.01, P-value = $9.4{\cdot}10^{-5}$). Alcohol consumption was associated also with a lower telomere length (β = −0.004, P-value = $7.5{\cdot}10^{-3}$) and Grim Age Principal Component (β = 0.13, P-value = $7.7{\cdot}10^{-4}$). Finally, marijuana consumption was not associated with any epigenetic clock.

Mediation between substance consumption and hypertension by CpG methylation

We evaluated whether the changes at the CpG methylation level mediated the effect of recreational drugs on hypertension. We first tested the association between smoking and hypertension. We considered that the group with the highest levels of smoking were those who smoked more than 11 cigarettes per day, joining the categories 11–20

Table 4. Top 15 differentially methylated CpG sites by marijuana consumption.

CpG	chr	position	Gene Symbol	Gene Group	On special occasions	Once per week	3–5 times per week	Regularly	F	P-Value	Adjusted P-Value
cg05107281	chr11	47072710	*CSTPP1*	Body	−0.004	−0.010	−0.014	0.001	9.4	1.6E−07	0.12
cg06499565	chr20	35374747	*NDRG3*	TSS1500	0.001	0.006	0.000	−0.001	8.7	5.7E−07	0.17
cg10054857	chr18	61816543	*LINC00305*	TSS1500	−0.004	−0.007	−0.002	−0.002	8.4	1.0E−06	0.17
cg24344693	chr10	133273964			−0.004	0.003	−0.001	−0.002	8.2	1.5E−06	0.17
cg20226924	chr5	133985272	*SEC24A*	Body	0.001	0.003	0.003	0.001	8.2	1.5E−06	0.17
cg05575921	chr5	373378	*AHRR*	Body	−0.003	−0.011	−0.012	−0.020	8.1	1.7E−06	0.17
cg21161138	chr5	399360	*AHRR*	Body	−0.004	−0.011	−0.009	−0.012	8.1	1.7E−06	0.17
cg19730404	chr9	139361517	*SEC16A; SEC16A*	ExonBnd; Body	−0.003	−0.001	0.001	−0.004	7.8	2.8E−06	0.25
cg19308363	chr12	116290566			0.006	0.005	−0.005	−0.015	7.7	3.4E−06	0.25
cg08258765	chr5	24841586	*LOC340107*	TSS1500	0.005	0.002	0.004	−0.002	7.7	3.8E−06	0.25
cg03838168	chrX	48433876	*RBM3*	Body	−0.003	−0.004	0.015	0.005	7.7	3.9E−06	0.25
cg16822035	chr13	113633379	*MCF2L; MCF2L*	Body; TSS1500	0.001	0.004	0.009	0.012	7.6	4.0E−06	0.25
cg11756734	chr12	9028945	*A2ML1*	3′UTR	−0.005	0.002	−0.007	0.003	7.5	4.8E−06	0.26
cg08415592	chr22	36648973	*APOL1*	TSS200	0.008	0.016	−0.004	0.007	7.4	5.8E−06	0.26
cg17325792	chr1	77042560	*ST6GALNAC3; ST6GALNAC3*	3′UTR; Body	0.003	0.001	0.003	−0.011	7.4	6.6E−06	0.26

Note: The CpG sites are annotated based on the chromosome (chr), the position (pos), the gene symbol from HGNC, and the gene group (based on the position of the CpG regarding the nearest gene). Each CpG site has a beta value for each consumer group vs non-consumers, a F-statistic (F), a nominal *p-value*, and an adjusted *p-value* by Bonferroni.

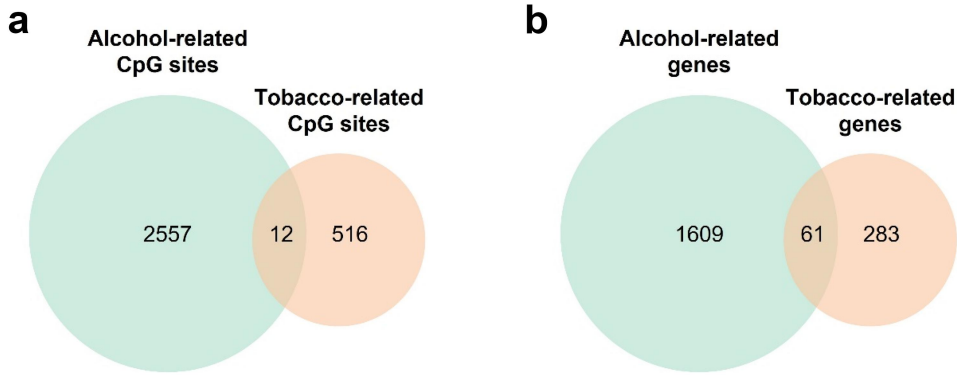

Figure 3. Venn diagrams comparing CpG sites and genes differentially methylated for tobacco and alcohol use. (a) Comparison between differentially methylated CpG sites with a P-value adjusted lower than 0.05. (b) Comparison between genes where the CpG sites differentially methylated are annotated.

and >20 cigarettes due to their low number of participants in each. We evaluated the association between smoking codified as numeric and high blood pressure as a binary variable and we did not detect a significant tendency (*P-value* = 0.26). The forest plot shows that the risk of hypertension increases with a higher number of cigarettes smoked except for the last group (more than 11 cigarettes per day) (Additional File 2: Fig. S5). Although we expected this group to be the one at the highest risk, we also observed that those participants were also the youngest (average of 5.75 years less, *P-value* = 0.005), suggesting a particularly strong healthy donor effect for this group. We tested the association after removing this group and we found a significant association between tobacco smoking and hypertension (*P-value* = 0.009, OR = 1.28). The forest plot in Figure 4a revealed a clear dose-response relationship where individuals who consume 6 to 10 cigarettes per day have 3.19 times of high blood pressure risk compared with non-smokers (*P-value* = 0.023). For the mediation analysis using CpG sites as mediators, we selected those CpG sites that were significantly associated with hypertension and tobacco smoking. The analysis revealed 10 CpG sites that were potential mediators. However, the univariate mediation analyses showed that none of those CpG sites was significantly mediating the effect of tobacco on hypertension with a FDR lower than 0.05 (Additional File 1: Table S4).

We also tested the association between marijuana consumption and high blood pressure. We did not find any significant association when comparing the 4 levels of consumption (from 'on special occasions' to 'regularly') with non-consumers (Additional File 2: Fig. S6).

As for alcohol use, we found a significant association between alcohol and higher blood pressure (*P-value* = 0.001, OR = 1.13). In addition, the forest plot in Figure 4b revealed a significant association between daily consumption with high blood pressure (*P-value* = 0.014, OR = 1.39) and a non-significant association between light to moderate consumption with the phenotype, as expected. Thus, we evaluated whether DNA methylation was mediating this association. We first performed logistic regression between the alcohol-related CpG sites and hypertension and found 76 significant CpG sites after correcting by multiple comparisons. To see the effect of each CpG site, we performed a univariate mediation analysis for each of these probes. Among them, 66 CpG sites significantly mediated the association between alcohol and hypertension with estimated proportions ranged between 3 and 71%. The most differentially methylated CpG site by alcohol consumption, cg06690548, was also the most significant mediator between alcohol consumption and high blood pressure after adjusting by covariates. This CpG site mediated 71% of the total effect of alcohol on the phenotype (FDR = 0.019). Furthermore, we observed that 12.8% of the hypertension variance was explained by alcohol consumption after adjusting by covariates. The variance explained was increased to 13.6% after including the cg06690548 methylation in the model, and to 20.5% after including the 66 significant CpG

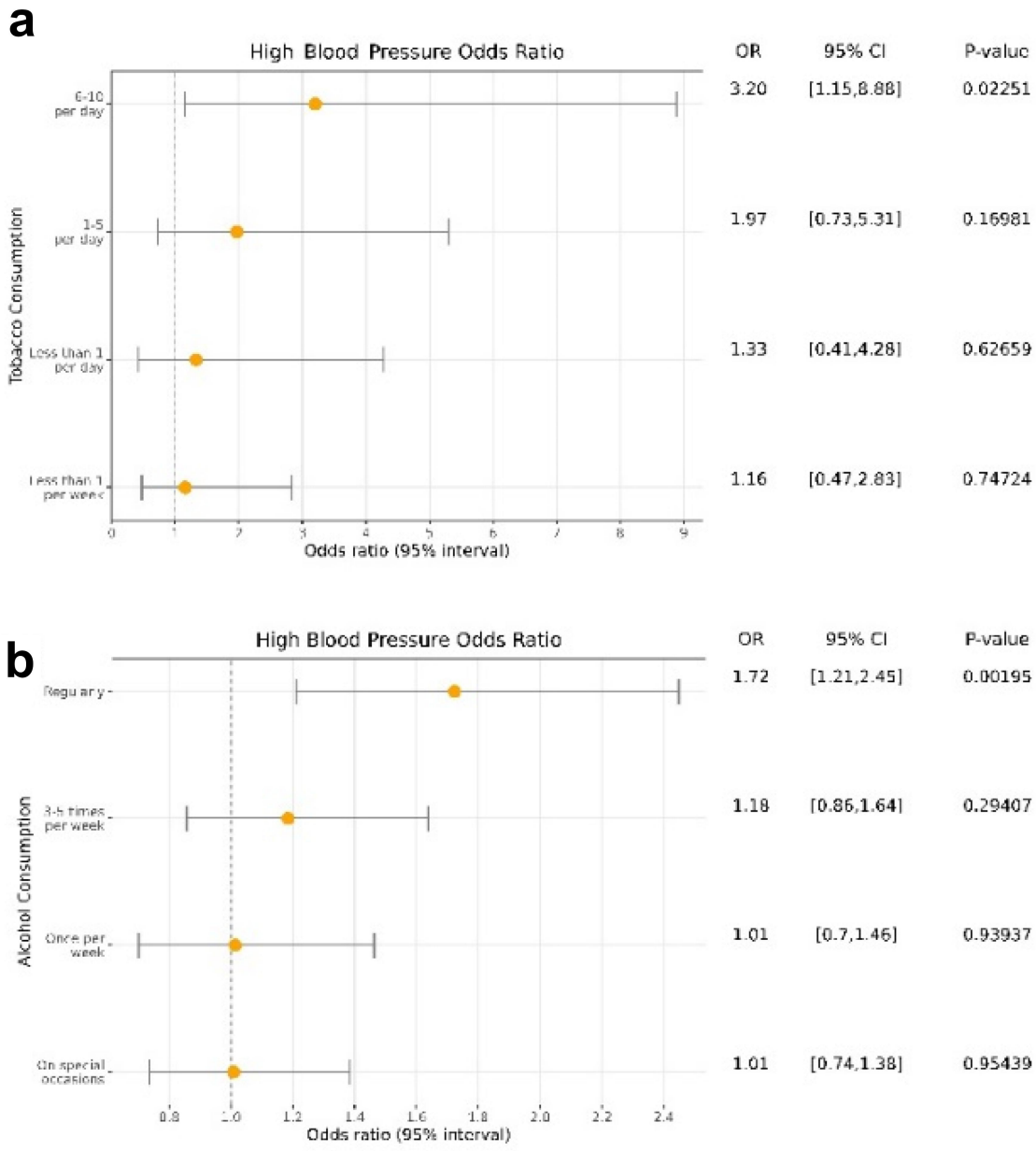

Figure 4. Forest plot of the association between tobacco (a) and alcohol (b) consumption with hypertension. OR: Odds Ratio.

sites. In these models, the association between hypertension and alcohol was no longer significant, further suggesting that methylation has an important mediation role in hypertension risk.

Discussion

The current study evaluated the effect of tobacco, alcohol, and marijuana consumption on genome-wide DNA methylation in 3,424 individuals from an EEUU population-based cohort. We identified 528 CpG sites differentially methylated according to tobacco smoking, 2,569 according to alcohol consumption, and no significant associations for marijuana consumption. Second, we detected a large overlapping between the differentially methylated genes by alcohol and tobacco consumption. Third, we found a significant mediation between alcohol consumption and hypertension by many alcohol-related methylation sites.

A considerable amount of literature has been published on DNA methylation changes due to smoking. The first studies evaluating these

changes were carried out in single genes or small panels of genes [63–65]. It was not until 2012 that the first epigenome-wide association study on tobacco was reported by Wan *et al.* [66]. Since that time, several studies have demonstrated the huge impact of tobacco on DNA methylation across the human genome, even in newborns when the tobacco exposure was during pregnancy [22,23,25–27,67,68]. Our findings were in line with previous research, demonstrating a large number of CpG sites differentially methylated along the genome. We were able to compare our results with the EWAS catalogue, and this revealed a high overlap in the CpG sites differentially methylated. Moreover, 98.9% of these CpG sites had the same direction of the effect. Among the probes that were not previously reported in the EWAS catalogue, it is remarkable the CpG site at *PHACTR2* gene, because it is involved in actin cytoskeleton organization and implicated in Parkinson's disease [69], and the CpG site at *GPX5* gene, since it protects cells and enzymes from oxidative damage. Additionally, our results confirm previous observations where the cg05575921 mapped to *AHRR* (*P-value* = $9.7{\cdot}10^{-221}$) and the cg21566642 in the 2q37.1 region (*P-value* = $5.9{\cdot}10^{-65}$) were the most significantly associated CpG sites to tobacco consumption [26–28]. Besides, the enrichment of the CpG sites differentially methylated revealed remarkable findings. Dopaminergic and cholinergic synapses are two systems that are affected by nicotine, the psychoactive substance found in tobacco. On one hand, nicotine activates the release of dopamine, inducing feelings of pleasure and reward [70]. This activation is one of the main reasons for which nicotine is so addictive. On the other hand, nicotine binds to receptors in the cholinergic synapse, enhancing the release of acetylcholine [71]. This neurotransmitter can improve cognition, attention, and memory. However, chronic exposure to nicotine can lead to a decrease of the numbers of receptors, which can produce cognitive impairment and other negative effects in the brain. Thus, the CpG sites differentially methylated by tobacco may be used as biomarkers to monitor addiction and neurotoxic effects on tobacco smokers.

Alcohol is known to affect DNA methylation. To date, several EWAS have detected CpG sites associated with alcohol consumption [29,31–33,72,73]. Here, we identified 2569 probes associated with drinking. As we found, many studies detected cg06690548 mapped at the *SLC7A11* promoter as the most alcohol-related methylation site [29,31,32]. Furthermore, Lohoff *et al.* demonstrated that various liver biomarkers were robustly associated with *SLC7A11* methylation status [31], suggesting an implication of this gene in the disturbance of the gastrointestinal system when consuming alcohol. In addition, we compared our results with those reported in study where they also performed an EWAS for alcohol consumption in 8,161 individuals. We found that 909 probes overlapped, all showing the same direction of effect. We observed that certain probes did not overlap, which could be attributed to differences in study design. Specifically, Lohoff et al used information on alcohol consumption during the previous week, while our study assessed general alcohol consumption habits. As a result, these discrepancies suggest that the timing and specificity of the alcohol consumption measure used in different studies can influence epigenetic changes. Besides, the genes differentially methylated in our study were highly enriched in autistic disorder, acquired scoliosis, curvature of the spine, small nose, and small midface. Engagingly, all these features are symptoms of the foetal alcohol spectrum disorder (FASD), which encompasses the range of adverse effects associated with alcohol exposure during pregnancy [74–77]. In addition, previous studies have already investigated the epigenetic mechanism linking autism and FASD [78]. Our results suggest that DNA methylation changes are important contributors to the relationship between alcohol consumption and FASD. Future studies should examine whether pregnant women with alcohol disorders could be monitored using epigenetic changes to prevent disorders in children.

The studies that have investigated DNA methylation modifications after marijuana consumption are limited. In 2015, Watson *et al.* evaluated in rats the effect of cannabis parental exposure on the epigenome of the nucleus accumbens [79] and they identified 1027 differentially methylated regions. Five

years later, Osborne *et al.* carried out the first EWAS on heavy cannabis consumption with and without tobacco comparing 48 consumers with 48 controls [35]. They found five differentially methylated sites in cannabis and tobacco users that replicated previous studies on the effects of tobacco. However, cannabis-only users had no evidence of significant differential methylation in any gene. Markunas *et al.* performed another EWAS with a larger sample size (1,247 ever users) consisting of women at risk of developing breast cancer [36]. They identified a unique significant CpG mapped to *CEMIP* 5' region. However, they designed a biomarker for lifetime cannabis use based on the top 50 EWAS CpG sites. In our study, 367 individuals smoked marijuana from occasionally to daily. The EWAS did not reveal significant CpG sites at the Bonferroni adjustment. Nonetheless, the genes where the 195 CpG sites with a *P-value* lower than $1{\cdot}10^{-4}$ were annotated were enriched in myelin assembly, essential for the proper functioning of the nervous system, as it enables the rapid and efficient transmission of nerve impulses between neurons. This suggests a possible implication of DNA methylation changes on the effects of THC, the active substance in marijuana, in cognitive and behavioural impairments. In addition, we detected cg05575921 (*AHRR*), the most significant tobacco-associated CpG site, differentially methylated according to marijuana use with a nominal *P-value* equal to $1.7{\cdot}10^{-6}$. Allen *et al.* already found that the link between marijuana use and epigenetic age acceleration was statistically mediated via hypomethylation at site cg05575921 [80]. This is consistent with the association of the *AHRR* gene with exposure to tobacco and fine particulate matter (PM2.5) which suggests that marijuana inhalation can produce similar effects [80,81].

In the current study, we aimed to investigate whether the adverse health effects commonly associated with the use of multiple substances, such as cardiovascular disease, could be attributed to epigenetic alterations. Our findings revealed a significant overlap of 12 CpG sites that displayed differential methylation levels in response to both tobacco and alcohol. Moreover, 61 genes overlapped between these two substances and were enriched in biological processes involved in the proper functioning of the nervous and cardiovascular systems. Therefore, similar epigenetic changes may explain the shared consequences of drug abuse. Targeting these specific pathways could be a strategy for preventing addiction, as well as neurological and cardiovascular disorders. In line with this, we found that tobacco and alcohol consumption were associated with shorter telomere lengths, as reported previously [82–84].

In our data, hypertension was partially associated with tobacco and highly associated with alcohol consumption, as demonstrated previously [39–42]. Marijuana was not associated with hypertension, in line with previous studies that have revealed ambiguous results [43–45]. In the case of tobacco exposure, we found unexpected results because the individuals who smoked the most were the ones who had less risk to develop hypertension. These results may be explained due to the lower age of the individuals in that group and also by the healthy donor effect of the data. This means that participants are volunteers who have paid for the TruAge test and may have healthy habits that protect them against hypertension although they are heavy smokers.

Our data replicated prior studies where light to moderate drinking was not associated with high blood pressure and heavy drinking increased the risk of the disease [8,40]. Another important finding was that 66 CpG sites significantly mediated the effect of alcohol consumption on hypertension. Importantly, the methylation levels of these probes increased the variance explained in hypertension by alcohol from 13.6% to 20.5%. More interestingly, lower methylation levels of cg06690548 at *SLC7A11*, cg18120259 at *LINC01512*, and cg19693031 at *TXNIP* have been seen previously associated with higher systolic and diastolic blood pressure [85–87]. Additionally, hypomethylation of cg06690548 was associated with higher expression of *SLC7A11* [87]. *SLC7A11* enhances antioxidant defence and protects against endothelial dysfunction and vascular inflammation. This increases vascular tone and rigidity, and consequently blood pressure. Also, Richard *et al.* evidenced triangular associations between methylation, gene expression, and blood pressure [86]. The univariate mediation based on cg06690548 methylation revealed that 70.5% of the effect of alcohol on high blood pressure was

mediated by the CpG methylation level (*P-value* = 0.006). In essence, we have demonstrated that the effect of heavy drinking on high blood pressure is partially mediated by the methylation of CpG sites that are significantly associated with the disease. This finding provides new insights on targets to prevent and manage hypertension in individuals with regular alcohol consumption.

The generalizability of these results is subject to certain limitations. First, DNA methylation was obtained from blood samples, thus, further research is required to understand the implication of the identified markers in each tissue. Second, genetics has an important role in substance use predisposition. In our analysis, we were not able to remove the genetic factor because of the lack of data. Some of the differentially methylated probes may be a consequence of the genetic differences and not the exposure itself. Notwithstanding this limitation, we filtered all the probes with a SNP in the extension base and all probes where 5bp 3'-subsequence overlapped with any of the SNPs with a global population frequency higher than 1%. Third, the consumption assessment was self-reported and not specific for a time period, limiting credibility and enhancing misclassification. In addition, we did not have information on whether marijuana was smoked mixed or not with tobacco. This information could benefit future studies on removing the tobacco effect. Fourth, we have compared our results with the EWAS catalogue for smoking and with a unique paper for alcohol consumption. It would be useful to compare our results with other published papers.

Our study also had notable strengths, including a large number of drinkers and a high variability in drinking frequency. This allowed us to test the mediation analysis between alcohol consumption and hypertension. Moreover, most studies are focused on evaluating the effects of one substance in drug-specific cohorts. Our data provided information on tobacco, alcohol, and marijuana consumption in the same individuals, along with clinical data. While marijuana consumption may be reported or suffer from healthy donor effect, we observed that fewer individuals smoked tobacco compared with marijuana, yet the effect of tobacco and alcohol on DNA methylation was strong. This suggests that marijuana may have a lower effect on blood DNA methylation compared to other substances. However, further studies with better consumption assessment are needed to confirm this observation.

Conclusions

To the best of our knowledge, this is the first study to assess simultaneously the effect of tobacco, alcohol, and marijuana on DNA methylation. We have shown that tobacco and alcohol have large effects on genome-wide DNA methylation, while marijuana consumption has small effects. Most importantly, many genes differentially methylated by smoking are also affected by alcohol consumption, suggesting a similar epigenetic impact after the consumption of recreational drugs. The results of this research also have significant implications for the understanding of how alcohol consumption increases hypertension. We demonstrated that 66 CpG sites were partially mediating the association between heavy drinking and hypertension and the most alcohol-related CpG site mediated 70.5% of this association. Finally, the current data highlight the importance of using blood methylation biomarkers in clinical practice to detect and monitor the adverse effects, such as addiction, derived from substance consumption.

Acknowledgments

We are grateful to all participants and researchers who took part in this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research has received funding from the Spanish Ministry of Science and Innovation through the "Centro de Excelencia Severo Ochoa 2019-2023 (CEX2018-000806-S) program, and support from the Generalitat de Catalunya through the CERCA Program. NC is supported by the Spanish regional program PERIS (Ref.: SLT017/20/000061), granted by Departament de Salut de la Generalitat de Catalunya. TruDiagnostics also provided funding for data analysis.

Data availability statement

The data that support the findings of this study are available upon reasonable request due to ensure the privacy of the participants. Please email varun@trudiagnostic.com for data requests. Any custom code or software used in our analysis is available at DOI: 10.5281/zenodo.6417926 (URL: https://zenodo.org/badge/latestdoi/296552532).

ORCID

Natàlia Carreras-Gallo http://orcid.org/0000-0001-8559-2313
Alejandro Cáceres http://orcid.org/0000-0001-8551-6695
Juan R Gonzalez http://orcid.org/0000-0003-3267-2146

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