Alcohol and Alcoholism, 2016, 51(3) 258–267
doi: 10.1093/alcalc/agv114
Advance Access Publication Date: 7 October 2015
Article

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Influence of Dopaminergic System Genetic Variation and Lifestyle Factors on Excessive Alcohol Consumption

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Received 17 December 2014; Revised 28 May 2015; Accepted 30 July 2015

Abstract

Aims: To examine the role of genetic and environmental factors in the pathogenesis of alcohol dependence in a Spanish cohort of women and men.

Methods: We analyzed the relationship between 56 genetic variants in 7 genes associated with the dopaminergic reward pathway and excessive alcohol consumption. The study sample ($N = 1533$, of which 746 were women) consisted of 653 heavy consumers and 880 very low consumers from the Spanish subcohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Single nucleotide polymorphisms (SNPs) were genotyped using a customized array. Lifestyle variables were also examined to assess associations between genetic and environmental factors.

Results: No statistically significant differences were found between cases and controls for the allele frequencies in five genes: *TH*, *SLC18A2*, *DRD1*, *DRD3* and *COMT*. Conversely, some alleles of the 12 SNPs from the *DRD2* locus and the 5 from the *MAOA* locus showed significant associations with excessive alcohol consumption. Namely, rs10891556 (*DRD2*) proved to be the only SNP positively correlated with excessive alcohol consumption in both sexes. *DRD2* rs1800497 and rs877138 were significantly associated in men, whereas *DRD2* rs17601612 and rs4936271 and *MAOA* rs5906898 were associated with excessive alcohol consumption in women. A correspondence analysis

provided an overall lifestyle profile of excessive drinkers, who were predominantly men who smoked, had large intakes of meat, small intakes of fruit and vegetables, whose jobs did not require high education levels and who engaged in little physical activity.

Conclusions: It has shown the influence of dopaminergic pathway in the genetics of alcohol dependence with differences between men and women and providing a lifestyle profile of excessive drinkers.

INTRODUCTION

Alcohol is a drug of abuse that depresses the central nervous system. The findings of recent twin studies have provided further support for the role of genetic factors in the pathogenesis of alcohol dependence, since higher concordance rates for monozygotic than for dizygotic twins were observed (Florez *et al.*, 2008). In addition, a GWAS meta-analysis with a large number of individuals has provided interesting information on novel genes that may regulate alcohol consumption (Schumann *et al.*, 2011).

The dopaminergic pathway plays a predominant role in the reward system, and several genetic studies on alcohol abuse have focused on analyzing the genes involved (Koob, 1992; Strat *et al.*, 2008; Le Foll *et al.*, 2009). Genetic factors may affect different stages of the dopaminergic pathway, namely: (a) enzyme-mediated dopamine synthesis; (b) internal transporter-mediated to the synaptic cleft; (c) the action of dopamine in the pre- and post-synaptic receptors; and (d) breakdown of dopamine by enzymes (Chen *et al.*, 2011). Accordingly, in this study we analyze several genetic markers related to the action of the neurotransmitter dopamine in the brain pathways that it modulates. The genes selected here were specifically involved in the encoding of enzymes implicated in the synthesis of dopamine (*TH*), internal transporter (*SLC18A2*), dopaminergic receptors (*DR1, DR2, DR3*) and enzymes associated with the degradation of dopamine (*MAO* and *COMT*).

The *TH* gene encodes the enzyme that catalyzes the conversion of L-tyrosine to dihydroxyphenylalanine (DOPA), the precursor of dopamine. The Val81Met polymorphism, rs6356 (Ludecke and Bartholome, 1995) is the most frequent known coding sequence variant of this gene. The hypothesis of the link between alcohol and other abuse substances and the dopamine-related reward system (Koob, 1996) supports its role as a candidate gene in these disorders (Dahmen *et al.*, 2005; Celorrio *et al.*, 2012).

SLC18A2 (also known as *VMAT2*) encodes a transporter of the monoaminergic neurons from the cytoplasm to the synaptic cleft (Lin *et al.*, 2005) described a haplotype in the promoter area of this gene with a protective role against alcohol excessive consumption, while Schwab *et al.* in 2005 (Schwab *et al.*, 2005) reported different polymorphisms which are associated with this disorder.

The first of the dopaminergic receptor genes here analyzed is *DRD1*. This gene plays an important role in modulating the cognitive processes of the pre-frontal cortex and it is involved in a number of brain functions like reward and reinforcement mechanisms (Rinaldi *et al.*, 2007). Several studies have reported an association between this gene and alcohol excessive consumption (Kim *et al.*, 2007; Batel *et al.*, 2008). The second dopaminergic receptor gene we evaluated in this study is *DRD2*, which has two widely studied polymorphisms: rs1799732 (known as -141C Ins/Del) and rs1800497 (known as 'TaqIA'). The first is located in the promoter region of *DRD2* and produces an insertion/deletion (indel) of a cytosine that is associated with the transcription of this receptor in the brain (Arinami *et al.*, 1997). The second is rs1800497, which was originally associated with reduced D2 receptor binding in all areas of the striatum (Thompson *et al.*, 1997; Pohjalainen *et al.*, 1998), but more recent findings indicate that it is located in exon 8 of gene *ANKK1* and is most probably associated with increased striatal activity of aromatic L-amino acid decarboxylase, the final enzyme in the biosynthesis of dopamine (Laakso *et al.*, 2005). The results reported in previous studies that have explored the association of *DRD2* with excessive alcohol consumption, however, have not been consistent (Wiesbeck *et al.*, 2006; Haberstick *et al.*, 2007; Hill *et al.*, 2008; Kovanen *et al.*, 2010). Finally, the last dopaminergic receptor we evaluated is the *DRD3* gene. Some studies have reported this to be a crucial receptor in mediating dopamine-dependent processes related to alcohol craving and relapse (Vengeliene *et al.*, 2007), while others have reported no association of *DRD3* with alcohol excessive consumption (Wiesbeck *et al.*, 2006; Kim *et al.*, 2007).

MAOA encodes the monoamine oxidase enzyme that plays a role in the metabolism of biogenic amines and neurotransmitters, including dopamine. An association between *MAOA* and alcohol dependence has been observed in some previous studies (Contini *et al.*, 2006; Nilsson *et al.*, 2011), although others did not report any associations (Mokrovic *et al.*, 2008).

The last gene we analyzed is *COMT*, which encodes the catechol-O-methyltransferase enzyme, responsible for degrading dopamine. There are well-known common functional variants in this gene, such as the SNP rs4680 (Lachman *et al.*, 1996). The enzymatic activity change mediated by this variant is ~40% higher between alleles (Chen *et al.*, 2004). Controversial results also have been also reported in studies of the association between *COMT* variants and alcohol excessive consumption (Tiihonen *et al.*, 1999; Foroud *et al.*, 2007).

Bearing in mind the physiological role of *TH, SLC18A2, DRD1, DRD2, DRD3, MAOA* and *COMT* in the brain's reward system modulated by the dopaminergic system, we assessed the potential association between these seven genes and the relative risk of alcohol excessive consumption, using 56 TagSNPs. As mentioned above, these genes have been the focus of numerous association studies that have produced controversial results. Our study is based on a broad sample from the Spanish subcohort of the European Prospective Investigation into Cancer (EPIC) cohort which includes a large number of women, and there have hitherto been few studies of women. Finally, we were able to capitalize on the rich data available in EPIC on diet, lifestyle and socio-demographic variables to explore relations between genetic and environmental factors with excessive alcohol consumption, because it is characterized by a problematic pattern of alcohol use leading to clinically significant impairment or distress, as manifested by multiple psychosocial, behavioral, or physiologic features. The pathogenesis of alcohol use disorder is not known, but its development may be the result of a complex interplay of genetics, environmental influences, specific personality traits and disorders of cognition. Aspects

of lifestyle balance, such as stable employment, relationships and accommodation, are likely to strengthen a person's resilience to prevent or overcome substance use difficulties. Low prevalence of self-reported low educational level was associated with, low regular exercise obesity, smoking, drinking, and poor self-reported health, although those with poor self-reported health the greatest increase of physical activity (Khaw *et al.*, 2008; Davies *et al.*, 2015).

METHODS

Sample and DNA extraction

The sample studied herein is taken from the Spanish EPIC (European Prospective Investigation into Cancer and Nutrition) project, a multicenter prospective cohort study involving 10 European countries (Riboli *et al.*, 2002; Bingham and Riboli, 2004). EPIC was designed to investigate the relationships between diet, nutritional status, lifestyle and environmental factors, and the incidence of cancer and other chronic diseases. Individuals of both sexes aged between 29 and 69 were recruited between 1992 and 1996 in the Spanish provinces of Asturias, Gipuzkoa, Navarra, Granada and Murcia. Most of the populations were blood donors or subgroups of the general population such as women attending breast cancer screening facilities. All individuals were of Caucasian origin. At the baseline survey, participants completed a detailed health and lifestyle questionnaire. Habitual physical activity was assessed using two questions referring to activity during the past year. Information on socio-demographic and lifestyle factors, including questions about educational attainment, tobacco use, lifetime alcohol consumption, reproductive history, physical activity, and medical history (medication use and history of diseases such as diabetes, cancer, and CVD), was obtained through an interviewer-administered lifestyle questionnaire (Epic Group of Spain, 1997; Buckland *et al.*, 2012). Each participant estimated their usual food intake over the previous year which was obtained through individual interviews at recruitment by using a validated electronic dietary history questionnaire that contained ~600 food items. The questionnaire was structured according to the different meals throughout the day, and participants were asked about what they ate and drank during these meals in a typical week of the year, taking into account seasonal variability. All of the foods consumed at least twice a month were registered. The portions of each food consumed (g/day) were quantified by using household measures, standard measures, and 35 sets of pictures with simple foods, food mixtures, and drinks. Oil added to the salads and cooked foods was also measured by using standard household measures. A food-composition table was used to calculate each participant's total energy intake (kcal/day) and daily nutrient intake. Anthropometric data were also collected at recruitment by measuring each participant's weight, height and waist/hip circumference.

For the purposes of defining the 'case' group, 'excessive alcohol consumption' was taken to mean an intake of >70 g/day for men and 42 g/day for women, following the criteria of Altisent and Cordoba (1993). The control group comprised individuals who were teetotalers or consumed less than one unit of alcohol per week (2 g/day). The sample was stratified by sex, age (±5 years) and center. Once certain individuals had been excluded due to drawbacks such as insufficient quantities of DNA and/or difficulties in the amplification and genotyping processes, the final study sample comprised a total of 1533 individuals of European ancestry, divided into 653 cases (402 men and 251 women) and 880 controls (385 men and 495 women). The mean age was 48.8 and 49.5 for cases and controls respectively.

Table 1 shows the main baseline characteristics of the analyzed population.

Information on diet, lifestyle and socio-demographic variables was collected following the procedure described in Munoz *et al.* (2012). Genomic DNA was extracted from frozen buffy coat, using FlexiGen kit columns, following the procedure described in Muñoz *et al.* (2012).

All participants signed informed consent forms. The protocol of the EPIC-Spain cohort study was approved by the Medical Ethical Committee of Hospital Universitario Donostia (03/08).

Table 1. Main characteristics of the analyzed population

Variables									
Sex		Men				Women			
Status		Controls		Cases		Controls		Cases	
	n	385	(%)	402	(%)	495	(%)	251	(%)
Age	Median (year-old)	51.0		51.3		47.1		46.6	
Smoking status	Never	161	41.8	61	15.2	337	68.1	130	51.8
	Former	100	26.0	103	25.62	55	11.1	30	11.9
	Current	124	32.2	238	59.2	103	20.8	90	35.9
	Unknown	–	–	–	–	–	–	1	0.4
Educational level	None	94	24.4	131	32.6	124	25.1	56	22.3
	Primary school	131	34.0	176	43.8	240	48.5	123	49.0
	Tech/Prof school	52	13.5	44	10.9	31	6.3	26	10.4
	Secondary school	45	11.7	26	6.5	45	9.1	21	8.4
	University	63	16.4	24	6.0	50	10.1	24	9.6
	Not specified	–	–	1	0.2	5	1.0	1	0.4
Physical activity	Inactive	83	21.6	68	16.9	30	6.1	24	9.6
	Moderately inactive	115	29.9	125	31.1	87	17.6	50	19.9
	Moderately active	123	31.9	144	35.8	336	67.8	153	60.9
	Active	64	16.6	65	16.2	42	8.5	24	9.6
Diet[a]	DQ ENDB Energy (kcal)	2417 (2104–2341)		3656 (3207–3932)		1899 (1597–2060)		2583 (2276–2841)	
	Vegetables (g/day)	246 (157–271)		251 (163–301)		236 (166–272)		241 (168–269)	
	Fruits, nuts and seeds (g/day)	379 (226–455)		219 (89–272)		359 (235–409)		222 (76–264)	
	Meat intake (g/day)	144 (110–165)		191 (147–213)		97 (73–115)		129 (97–151)	

[a]Values are medians and 33 and 67% percentiles.

SNP selection criteria and genotyping

TagSNPs from genes *TH, SLC18A2, DRD1, DRD2, DRD3, MAOA* and *COMT* were selected on the basis of information compiled by the HapMap project (http://www.hapmap.org) for populations of European ancestry (phase II CEU population, release 24 based on dbSNP version 126 and NCBI genome build 36). For each gene a list of SNPs between 50 kb upstream and 30 kb downstream was established. This list was later used to define the haplotype blocks from a minimum of 10 kb upstream to at least 3 kb downstream of the gene of interest, using the default parameters in Haploview v4.2 software (Barrett *et al.*, 2005) and based upon the method described by Gabriel *et al.* (2002). The haplotype tagging SNPs were selected by use of the Tagger algorithm, also as implemented in Haploview. Parameters used for TagSNP selection were minor allele frequency (MAF) ≥5% in individuals of European ancestry, minimum disequilibrium coefficient ($r^2 \geq 0.8$) between each pair of tagged and TagSNPs (pairwise tagging), and SNPs tagging haplotypes with a frequency of ≥5%. Thus, 63 TagSNPs were selected from the 7 genes involved: 10 for *TH*, 10 for *SLC18A2*, 3 for *DRD1*, 15 for *DRD2*, 8 for *DRD3*, 5 for *MAOA* and 12 for *COMT*. Genotyping was performed using the Taqman OpenArray Genotyping System technology (Applied Biosystems, Foster City, CA, USA). As an internal quality control 79 samples (~5% of the total sample) were genotyped in duplicate: the concordance in the results was >99.5%.

Diet, alcohol and lifestyle

To explore potential associations between genetic and environmental factors and excessive alcohol consumption, additional information on diet, lifestyle and socio-demographic variables was collected following the procedure described in Muñoz *et al.* in 2012.

Statistical analysis

Genetic data obtained were analyzed following the procedure described in Munoz *et al.* (2012). Genetic association analyses to study the main effects of each variant on excessive alcohol consumption were performed under the additive (per allele) genetic inheritance model. To adjust for multiple testing, the conventional $\alpha = 0.05$ was divided by the number of independent loci analyzed (0.05/7). Thus, the statistical significance level was set at a nominal $P < 0.05$ and an adjusted *P*-value of 0.007.

A logistic regression analysis was carried out in order to compare the genotypes frequency in cases and controls. We estimated the odds ratio (OR) and 95% confidence interval (CI). All models were adjusted for age (±5 years) and sex.

The deviations of the genotype frequencies from those expected under Hardy–Weinberg equilibrium (HWE) were assessed by the Pearson's chi-squared test, as implemented in the SNPassoc R package (Gonzalez *et al.*, 2007) in the control and cases groups. The case-control excessive alcohol has 80% power to detect variants. The EM algorithm, also implemented in the SNPassoc 'R' package, was used to analyze linkage disequilibrium (LD) between SNPs in each gene region and to infer haplotype frequencies. The association between excessive alcohol intake and gene haplotypes with a frequency greater than 0.01 was also measured by logistic regression using the most frequent haplotype in the control group as the referent category.

A Pearson's correlation coefficient was used to assess the correlation between SNP frequencies in the control group and the frequencies reported for the Caucasian population (CEU) in the HapMap database (Supplementary Fig. S1).

Independence or association between the socio-demographic and lifestyle variables considered in our study was assessed by calculating Spearman's rank-order correlation coefficients. Likewise, a correspondence analysis (CA) was performed to identify potential associations of genetic and environmental factors with excessive alcohol consumption. CA is a multivariate statistical technique usually applied to contingency tables with the aim of constructing a Cartesian diagram based on the association of the variables examined. In the resultant graph, the different modalities of the contingency table are represented jointly, so that the proximity between the points is assumed to be related to the level of association between the cited modalities. CA is an analogue of principal component analysis, which is appropriate for discrete rather than continuous variables (Hill, 1974). This analysis was performed using the SPSS v17.0 statistical package (SPSS Inc., Chicago, IL, USA), and the results are represented in the two-dimensional space delimited by the first two axes.

RESULTS

A total of 63 SNPs were initially selected for this study, though 7 of them were ruled out due to failures in amplification or because the separation between the genotype clusters was insufficient. Information about the SNPs and the genetic coverage of each gene region can be found in Table 2. Therefore, 56 SNPs (89%) were successfully genotyped: 8 for *TH*, 9 for *SLC18A2*, 3 for *DRD1*, 12 for *DRD2*, 7 for *DRD3*, 5 for *MAOA* and 12 for *COMT* (Table 3). As observed in Table 3, no significant departure from Hardy–Weinberg equilibrium was detected for any of the SNPs examined (all *P*-value > 0.001). The allele frequencies of the 56 SNPs included in subsequent analyses was very similar between our control group and in the CEU population from HapMap (Supplementary Fig. S1), as revealed by the highly significant association detected for the Pearson correlation coefficient ($R = 0.95$; $P < 0.001$).

Allele frequencies for each SNP in cases and controls, as well as odds ratios and *P*-values of the association with excessive alcohol consumption for both men and women are also summarized in Table 3.

Table 2. SNPs and haplotypes of the seven genes here studied

Genes	SNPs Selected	SNPs Genotyped	Genetic variation coverage (%)	Haplotype blocks	% of tagged haplotypes	Association to excessive alcohol intake
TH	10	8	61	2	60–80	(−)
SLC18A2	10	9	31	3	92–92–78.6	(−)
DRD1	3	3	99	1	99	(−)
DRD3	8	7	38	2	88–89	(−)
COMT	12	12	39	2	85–86	(−)
DRD2	15	12	34	2	82.3–62.2	(+)
MAOA	5	5	72	1	95	(+)

Table 3. Analyzed SNPs in the *TH, SLC18A2, DRD1, DRD2, DRD3, MAOA y COMT* loci, genotyping results and association with excessive alcohol intake by sex

SNP	Alleles	Gene	Haplotype blocks	Chr.	Cases (*n*)	Controls (*n*)	HWE	MAF CEU HapMap	Men				Women			
									Cases (MAF)	Controls (MAF)	OR (95% CI)	*P*-value	Cases (MAF)	Controls (MAF)	OR (95% CI)	*P*-value
rs2070762	A/G	*TH*	Single	11	640	845	0.240	0.483	0.489	0.458	1.10 (0.83–1.46)	0.520	0.494	0.485	0.95 (0.73–1.24)	0.711
rs10840489	C/T		Block 1	11	640	864	0.634	0.100	0.110	0.114	0.99 (0.63–1.56)	0.972	0.134	0.127	0.95 (0.65–1.40)	0.807
rs10770141	G/A		Block 2	11	642	860	0.941	0.433	0.350	0.356	0.90 (0.67–1.20)	0.456	0.330	0.376	0.83 (0.62–1.11)	0.209
rs10840491	G/A			11	638	852	0.603	0.092	0.102	0.103	1.30 (0.80–2.12)	0.287	0.122	0.117	0.97 (0.65–1.45)	0.884
rs4929966	C/G			11	643	857	0.359	0.308	0.214	0.245	0.87 (0.62–1.21)	0.407	0.192	0.247	0.77 (0.54–1.08)	0.132
rs11042982	G/C			11	643	855	0.264	0.108	0.125	0.137	1.22 (0.79–1.89)	0.378	0.155	0.148	1.01 (0.71–1.45)	0.939
rs11564709	C/T			11	630	846	0.058	0.144	0.039	0.069	0.62 (0.33–1.19)	0.153	0.045	0.071	0.65 (0.37–1.13)	0.118
rs7129483	C/T			11	639	846	0.015	0.309	0.223	0.239	0.79 (0.55–1.13)	0.192	0.232	0.274	0.88 (0.63–1.23)	0.465
rs2803825	G/A	*SLC18A2*	Block 1	10	636	856	1.000	0.108	0.037	0.053	0.81 (0.39–1.67)	0.564	0.032	0.046	0.62 (0.30–1.29)	0.193
rs2803823	G/A			10	613	837	0.459	0.067	0.085	0.075	1.22 (0.71–2.10)	0.473	0.086	0.075	1.12 (0.68–1.86)	0.649
rs363371	G/A			10	650	871	0.899	0.258	0.140	0.178	0.86 (0.58–1.27)	0.445	0.152	0.145	0.92 (0.63–1.34)	0.657
rs363393	A/T			10	648	870	0.336	0.167	0.202	0.199	1.21 (0.84–1.75)	0.292	0.202	0.197	1.06 (0.77–1.45)	0.727
rs363395	G/A			10	651	875	1.000	0.050	0.026	0.009	1.99 (0.65–6.08)	–	0.018	0.020	0.87 (0.33–2.32)	–
rs929493	T/C		Block 2	10	644	858	0.484	0.175	0.178	0.182	0.80 (0.54–1.17)	0.246	0.163	0.173	0.80 (0.56–1.15)	0.224
rs363222	G/C			10	645	864	0.022	0.283	0.328	0.326	0.89 (0.66–1.20)		0.308	0.307	0.94 (0.71–1.25)	0.662
rs363226	C/G		Block 3	10	626	851	0.641	0.367	0.347	0.329	1.09 (0.80–1.49)	0.563	0.362	0.330	1.23 (0.92–1.63)	0.163
rs363230	T/C			10	647	869	0.331	0.467	0.416	0.422	0.98 (0.73–1.30)	0.877	0.424	0.431	1.03 (0.78–1.37)	0.815
rs4867798	T/C	*DRD1*	Block 1	5	645	853	0.691	0.263	0.321	0.314	0.90 (0.66–1.21)	0.482	0.319	0.311	1.05 (0.78–1.42)	0.733
rs686	A/G			5	642	867	0.175	0.405	0.354	0.385	0.84 (0.63–1.13)	0.249	0.366	0.389	0.88 (0.67–1.16)	0.366
rs5326	C/T			5	642	871	0.188	0.133	0.115	0.111	1.21 (0.76–1.91)	0.423	0.110	0.121	0.90 (0.59–1.37)	0.616
rs11604671	A/G	*DRD2*	Block 1	11	647	864	0.837	0.474	0.493	0.477	1.23 (0.91–1.66)	0.170	0.477	0.464	1.13 (0.86–1.48)	0.375
rs1800497	G/A			11	647	875	0.458	0.225	**0.208**	**0.154**	**1.68 (1.15–2.45)**	**0.005**	0.204	0.169	1.25 (0.90–1.75)	0.187
rs2587550	A/G			11	649	873	0.810	0.308	0.284	0.319	0.81 (0.59–1.11)	0.195	0.270	0.292	0.94 (0.70–1.28)	0.706
rs6277	A/G			11	645	871	0.327	0.466	0.429	0.418	1.13 (0.83–1.55)	0.431	0.427	0.406	1.18 (0.89–1.56)	0.245
rs1076563	C/A			11	646	858	0.938	0.342	0.348	0.328	1.12 (0.82–1.54)	0.472	0.341	0.328	1.18 (0.89–1.57)	0.250
rs11214606	C/T			11	649	874	0.556	0.058	0.022	0.028	0.71 (0.30–1.66)	–	0.040	0.031	1.06 (0.54–2.11)	0.858
rs17601612	G/C		Block 2	11	649	872	0.220	0.417	0.429	0.432	0.91 (0.68–1.21)	0.510	**0.418**	**0.475**	**0.76 (0.58–0.98)**	**0.036**
rs4936271	T/C			11	648	864	0.631	0.458	0.500	0.485	1.17 (0.88–1.55)	0.282	**0.488**	**0.441**	**1.36 (1.04–1.76)**	**0.021**
rs7131056	C/A			11	626	829	0.881	0.442	0.372	0.383	0.83 (0.61–1.12)	0.217	0.373	0.359	1.03 (0.78–1.36)	0.852
rs10891556	G/T			11	650	869	0.201	0.158	**0.185**	**0.165**	**1.54 (1.04–2.29)**	**0.027**	**0.202**	**0.152**	**1.55 (1.09–2.20)**	**0.014**
rs1799732	C/–		Single	11	626	853	0.823	0.050	0.117	0.147	0.78 (0.45–1.37)	0.393	0.119	0.160	0.75 (0.46–1.24)	0.261
rs877138	A/G		Single	11	640	861	0.503	0.375	**0.327**	**0.295**	**1.49 (1.08–2.06)**	**0.013**	0.302	0.273	1.14 (0.85–1.52)	0.382
rs2399496	A/T	*DRD3*	Block 1	3	626	832	0.051	0.500	0.484	0.491	1.00 (0.75–1.33)	0.996	0.487	0.451	1.20 (0.92–1.56)	0.179
rs2134655	C/T			3	650	872	0.507	0.275	0.268	0.264	1.16 (0.84–1.60)	0.370	0.260	0.303	0.86 (0.63–1.15)	0.305
rs324035	C/A			3	647	859	0.570	0.200	0.182	0.192	0.77 (0.54–1.10)	0.159	0.198	0.178	0.99 (0.70–1.40)	0.947
rs167770	A/G		Block 2	3	621	822	0.794	0.292	0.265	0.273	0.92 (0.66–1.27)	0.602	0.269	0.278	1.14 (0.83–1.57)	0.420
rs11706283	C/T			3	639	867	0.169	0.092	0.076	0.064	1.48 –(0.83–2.64)	0.1763	0.073	0.095	1.01 (0.61–1.65)	0.973
rs10934256	C/A			3	650	871	0.519	0.200	0.192	0.196	0.90 (0.63–1.30)	0.582	0.182	0.195	1.05 (0.74–1.48)	0.799
rs6280	T/C			3	645	869	0.744	0.350	0.278	0.298	0.82 (0.60–1.11)	0.199	0.280	0.293	1.00 (0.73–1.36)	0.995

rs5906898	G/A	*MAOA*	Block 1	X	648	872	0.400	0.210	0.211	0.244	1.25 (0.77–2.04)	–	**0.220**	**0.246**	**0.65 (0.47–0.90)**	**0.008**
rs1181275	C/T			X	649	856	0.078	0.111	0.082	0.100	0.73 (0.36–1.5)	–	0.080	0.088	0.87 (0.55–1.37)	0.552
rs5906957	G/A			X	651	871	0.890	0.178	0.190	0.200	1.23 (0.73–2.07)	–	0.189	0.203	0.72 (0.51–1.01)	0.053
rs3027392	G/A			X	650	870	0.680	0.067	0.054	0.036	0.49 (0.17–1.46)	–	0.050	0.059	0.95 (0.52–1.73)	0.866
rs3027399	G/C			X	645	864	0.280	0.070	0.070	0.076	0.91 (0.42–1.96)	–	0.094	0.069	1.35 (0.85–2.14)	0.208
rs5993875	G/A	*COMT*	Block 1	22	643	866	0.942	0.433	0.350	0.414	0.88 (0.65–1.19)	0.404	0.416	0.366	1.17 (0.89–1.54)	0.254
rs7287604	C/T			22	628	819	0.085	0.167	0.208	0.202	0.83 (0.58–1.20)	0.332	0.215	0.206	0.94 (0.67–1.34)	0.743
rs9606186	G/C			22	649	873	0.776	0.490	0.351	0.412	0.89 (0.66–1.20)	0.461	0.415	0.373	1.14 (0.87–1.50)	0.338
rs9605030	C/T			22	646	866	0.086	0.117	0.181	0.150	1.13 (0.77–1.67)	0.526	0.146	0.178	0.78 (0.54–1.12)	0.170
rs9605031	C/T			22	644	857	0.344	0.317	0.348	0.290	1.25 (0.92–1.70)	0.156	0.292	0.337	0.88 (0.66–1.18)	0.393
rs16982844	C/A			22	648	869	1.000	0.020	0.023	0.030	0.68 (0.30–1.54)	0.364	0.034	0.038	0.90 (0.45–1.82)	0.772
rs4646312	T/C		Block 2	22	649	870	0.890	0.431	0.439	0.425	1.05 (0.78–1.40)	0.752	0.410	0.441	0.95 (0.73–1.25)	0.732
rs4633	C/T			22	629	827	0.675	0.475	0.450	0.474	0.89 (0.66–1.20)	0.445	0.483	0.456	1.11 (0.85–1.47)	0.441
rs4680	G/A			22	648	864	0.632	0.483	0.452	0.474	0.90 (0.67–1.20)	0.462	0.490	0.457	1.08 (0.82–1.41)	0.596
rs4646316	C/T			22	625	820	0.016	0.217	0.270	0.285	0.94 (0.68–1.30)	0.695	0.262	0.287	0.91 (0.66–1.25)	0.563
rs165774	G/A			22	648	862	0.871	0.342	0.280	0.296	0.97 (0.70–1.35)	0.872	0.284	0.307	0.82 (0.62–1.09)	0.166
rs174696	T/C			22	646	859	0.758	0.192	0.186	0.213	0.75 (0.51–1.09)	0.135	0.209	0.209	1.04 (0.75–1.43)	0.824

The HWE was analyzed in the group of controls

Comparisons between cases and controls in 6 out of the 56 SNPs (*DRD2* rs1800497, rs17601612, rs4936271, rs10891556 and rs877138 and *MAOA* rs5906898) show statistically significant main effects with nominal $P < 0.05$ whereas only one SNP in gene *DRD2* (rs1800497) was statistically significant after adjustment for multiple comparisons ($P < 0.007$).

None of the SNPs or haplotypes of the following genes *TH, SLC18A2, DRD1, DRD3* and *COMT* showed any significant association with excessive alcohol consumption in men or women.

On the other hand, the following polymorphisms of the *DRD2* and *MAOA* genes showed significant association with excessive alcohol consumption in men or women.

DRD2

The association results (Table 3) indicate that several SNPs in *DRD2* exhibited significant differences in allele frequencies between cases and controls in men and proved to be risk factors for excessive alcohol consumption: rs1800497 (OR = 1.68, 95% CI: 1.15–2.45, $P = 0.005$), rs10891556 (OR = 1.54, 95% CI: 1.04–2.29, $P = 0.027$), and rs877138 (OR = 1.49, 95% CI: 1.08–2.06, $P = 0.013$). In addition, the 'GAAGAC' haplotype in block 1 (OR = 1.92, 95% CI: 1.20–3.08, *P*-value = 0.007) and the 'GCCT' in block 2 (OR = 1.55, 95% CI: 1.01–2.38, $P = 0.043$), that contain rs1800497 and rs10891556 variants respectively, also showed significant association with alcohol use in males (Supplementary Table S1). The association disappeared after adjustment which it can be inferred indicating this association is caused mainly by the effect of these individual SNPs, or any other SNP being in LD. In the same way, comparisons between cases and controls in the women subgroup for rs17601612 (OR = 0.76, 95% CI: 0.58–0.98, $P = 0.036$), rs4936271 (OR = 1.36, 95% CI: 1.04–1.76, $P = 0.021$) and rs10891556 (OR = 1.55, 95% CI: 1.09–2.20, $P = 0.014$) showed statistically significant association with excessive alcohol consumption. Likewise, haplotype 'GCCT' in block 2 (OR = 1.74, 95% CI: 1.18–2.55, $P = 0.005$) was associated with to be a risk factor for excessive alcohol consumption in women.

MAOA

Interestingly, SNP rs5906898 (OR = 0.65, 95% CI: 0.47–0.90, $P = 0.008$) was inversely associated with against excessive alcohol consumption in women. Association analysis with *MAOA* haplotypes did not yield more information than single-SNP analysis, 'ACAGG' (OR = 0.66, 95% CI: 0.45–0.96, $P = 0.031$), statistical significance disappeared when the haplotype association analysis was adjusted for SNP rs5906898, suggesting that this SNP (rs59006898) or any in LD with it was responsible for the association.

Spearman rank correlation coefficients for the targeted variable 'status' (excessive alcohol consumption) and nine other biological, environmental and lifestyle parameters included in the study are shown in Supplementary Table S2. Age, physical activity and consumption of vegetables were not associated with the targeted variable. Of the parameters significantly associated ($P < 0.001$) with status, smoking status, energy intake and meat intake were positively correlated whereas sex, education level and consumption of fruit were negatively correlated. To explore potential associations of biological, environmental and lifestyle variables with genetic data (SNP allele frequencies), a CA was performed. The results of the CA are represented in a Cartesian diagram (Fig. 1). The first two axes accounted for >30% of the total variance (axis 1: 15.74%, axis 2: 14.30%). The positions of the variables included in the CA with respect to axis 2 provided a clear summary of the associations between the different parameters. For instance an inverse association can be observed between the variables sex (which plotted at the negative end of axis 2) and status, positioned in the positive segment of axis 2. Overall, axis 2 represents a gradient that runs from low values for sex (women 1; men 2) to high values for status (controls 1; cases 2). A differential association for sex and status can thus be inferred with the various lifestyle and environmental variables: Education level, consumption of fruit and vegetables and

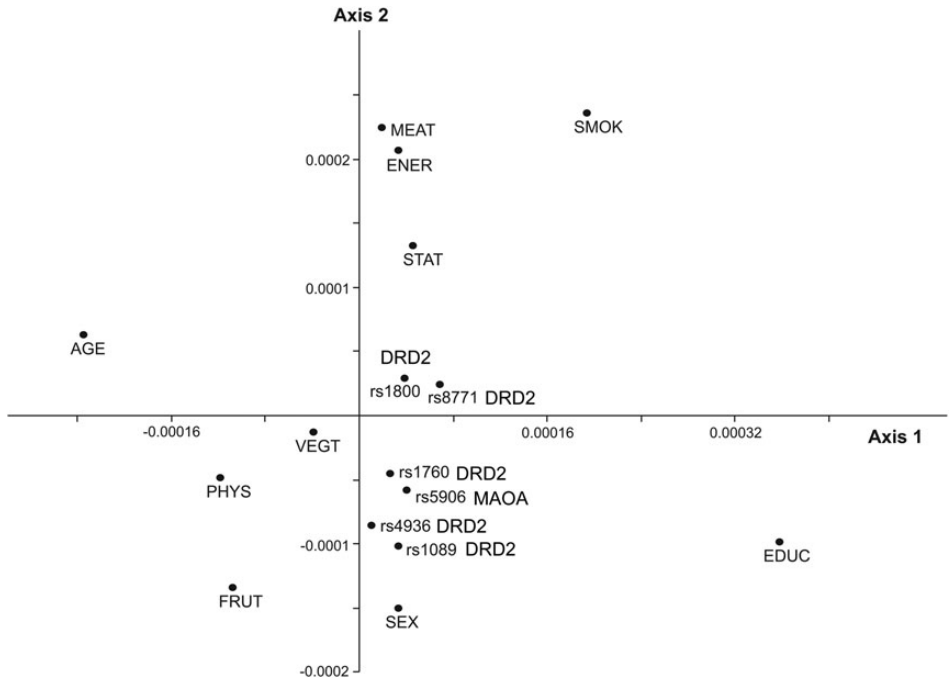

Fig. 1. Cartesian diagram of correspondence analysis for studying genetic and environmental factors with excessive alcohol consumption.

physical activity were more associated with women in the control group, while smoking status, energy intake and meat intake were mostly associated with men identified as cases. From the genetic viewpoint, the SNPs that showed the closest association with status were rs1800497 and rs877138 (*DRD2*), which also plotted in the quadrant delimited by the positive segments of axis 1 and 2, mirroring their significant association with alcohol intake only in men. Likewise, the SNPs that showed significant associations with status only in the women subsample (rs17601612 and rs4936271 from *DRD2*, and rs5906898 from *MAOA*) appeared clustered in the right lower quadrant, as expected. Thus, axis 2 seems to essentially represent the association of traditional gender differences in lifestyle with alcohol consumption, as well as the particular association of the SNPs with alcohol excessive consumption depending on sex.

DISCUSSION

This study examined the effect/influence of genetic factors on excessive alcohol consumption by studying 56 TagSNPs belonging to seven genes (*TH*, *SLC18A2*, *DRD1*, *DRD2*, *DRD3*, *MAOA* y *COMT*) related to the dopaminergic reward pathway. Each gene was analyzed by TagSNPs to define haplotypes effectively. However, the haplotype analysis of these genes did not provide additional information because each of the haplotypes associated with alcohol consumption contained the associated variant in the single-SNP analysis.

Five of the seven genes examined (*TH*, *SLC18A2*, *DRD1*, *DRD3* and *COMT*) showed no significant association with excessive alcohol consumption. *TH* has been previously analyzed in relation to alcohol and nicotine addiction, and a positive association has been found (Ludecke and Bartholome, 1995; Ishiguro *et al.*, 1998; Anney *et al.*, 2004; Dahmen *et al.*, 2005; Celorrio *et al.*, 2012). Similarly, many studies have reported associations for some polymorphisms of *SLC18A2* (rs363387 and rs363333) with the development of alcohol dependence (Schwab *et al.*, 2005; Eiden and Weihe, 2011). Likewise, Lin *et al.* (2005) suggested the existence of a haplotype that decreases susceptibility to alcohol excessive consumption. Regarding *DRD1* our findings do not coincide with those reported in earlier studies (Kim *et al.*, 2007; Batel *et al.*, 2008) in which the SNP rs686 was reported to be associated with alcohol excessive consumption. These discrepancies may be related to differences in sample selection, since the critical value for inclusion in our study was 70 g/day for men and 42 g/day for women, while previous studies have used different levels or other criteria for selecting samples, including controls, e.g. tests such as DSM-IV and AUDIT; these tests are not performed on our sample because there are no alcoholic addictive characteristics. Nor did gene *DRD3* show any significant association with alcohol excessive consumption, coinciding with the findings of previous studies (Wiesbeck *et al.*, 2006; Kim *et al.*, 2007). Likewise the *COMT* enzyme showed no significant association in men or women. SNP rs4680, one of the most widely analyzed to date, has revealed contradictory information. It has been reported to be significantly associated with alcohol excessive consumption and other pathologies such as schizophrenia (Tiihonen *et al.*, 1999; Enoch *et al.*, 2009; Costas *et al.*, 2011). However, other studies have shown that *COMT* is not associated with alcohol drinking or smoking (Foroud *et al.*, 2007). This is borne out by our findings. As Ioannidis *et al.* (2001) stablished, the first study often suggests a stronger genetic effect than is found by subsequent studies. Both bias and genuine population diversity might explain why early association studies tend to overestimate the disease protection or predisposition conferred by a genetic polymorphism.

On the other hand, significant differences were found between cases and controls for some SNPs in the *DRD2* and *MAOA* genes. The analysis of the twelve SNPs in the *DRD2* gene showed an association with excessive alcohol consumption for five: rs1800497, rs10891556 and rs877138 in men, and rs17601612, rs4936271 and rs10891556 in women.

SNP rs1800497 was the only SNP associated with alcohol excessive consumption at a highly significant level (adjusted P-value < 0.007) in men. This SNP is located 10,541 bp downstream of the termination codon of the *DRD2* gene, in exon 8 of *ANKK1*, which encodes a kinase enzyme (Neville *et al.*, 2004). According to Laakso *et al.* (2005), rs1800497 could affect the striatal activity of aromatic L-amino acid decarboxylase, the final enzyme in the biosynthesis of dopamine. But it is not known whether the kinase encoded by *ANKK1* influences the expression or function of the *DRD2* gene, though there is evidence that it is not expressed in the brain (Fossella *et al.*, 2006), which supports the idea that the polymorphism rs1800497 or any in LD with it could modify the expression of *DRD2*. Wang *et al.* (2013) performed a large-scale meta-analysis that confirmed the association between the rs1800497 and alcohol excessive consumption. However, sexual dimorphism was not evaluated. Future studies on the functional significance of the ANKK1/DRD2 Taq1A polymorphism in alcohol excessive consumption may provide new clues to explain the differences between sexes. In a similar manner, other SNPs that showed significant associations ($P < 0.05$) with high excessive alcohol consumption in *DRD2* were rs10891556 and rs877138 in men and rs10891556, rs17601612 and rs4936271 in women.

SNP rs10891556 was the only polymorphism significantly associated with excessive alcohol consumption in both sexes. It is a TagSNP located upstream from the gene that tags five SNPs in the same area, which could interfere the gene transcription. This polymorphism has been described previously in a study by Sangrajrang *et al.* in 2010 (Sangrajrang *et al.*, 2010) where alcohol consumption was associated with an increased risk of breast cancer.

SNP rs877138, located downstream from *DRD2* and upstream from *ANKK1* that tags seven SNPs, five of which are located in intronic areas of *ANKK1*, whereas the other two (rs4938013 and rs17115439) are functional SNPs located in exon 2 of *ANKK1* that produce silent mutations with changes in Ile151Ile and Ser85Ser respectively. SNP rs4938013 has been reported to be located within a haplotype significantly associated with nicotine dependence (Gelernter *et al.*, 2006). Thus, SNP rs877138 or any other SNP in LD with it, such as the silent mutations of rs4938013 and rs17115439 could alter the expression of *DRD2* and modulate the response to excessive consumption of alcohol.

The *DRD2* SNPs rs17601612 and rs4936271 showed positive associations only in women. The first is a SNP that may protect against excessive alcohol consumption and tags an additional ten SNPs located upstream and in intronic areas of the gene. SNP rs17601612 has been analyzed in relation to delirium caused by alcohol withdrawal, although no correlation was found (van Munster *et al.*, 2010). No information from previous studies has been found for rs4936271, which tags four SNPs located upstream from the gene. Therefore, more studies are necessary to confirm our results.

To date the most widely analyzed polymorphism of *MAOA* in regard to the association with alcohol excessive consumption is the 30 base pair repeat polymorphism (VNTR) located in the 5′-untranslated region. This polymorphism, which may alter transcriptional efficiency, has been associated with alcohol excessive consumption in several previous studies (Huang *et al.*, 2004; Contini *et al.*, 2006; Nilsson

et al., 2011). However, the meta-analysis of Forero *et al.* (2015) did not support consistent associations with *MAO-A* in men. The TagSNP rs5906898 and the haplotype of which it is a part showed statistically significant association with excessive alcohol consumption in women. Since *MAOA* is located on the X-chromosome, there is significant linkage disequilibrium between its SNPs. Thus, rs5906898 tags more than 30 SNPs, of which rs1137070 results in the silent mutation Asp470Asp and rs6323 results in the silent mutation Arg297Arg, which could be influential in the protein translation.

The results of the gene-environment interaction analysis also revealed dissimilarities in biological, environmental and lifestyle variables between both sexes. The rate of excessive alcohol consumption is greater among males (12.4% among men, 4.9% among women) (Hasin *et al.*, 2007). For example, women appear to be less likely than men to manifest certain risk factors for alcohol use and problems and are more likely to have factors that may protect against these problems (Nolen-Hoeksema, 2004). Therefore, genetic factors may play a different role on alcohol consumption patterns (excessive alcohol consumption) in women and in men as our study suggests.

Overall, the representation of axes 1 and 2 of the CA provides an overall picture of the predominant profile of the 'cases', which prove to be mainly men who smoke, eat large quantities of meat and little fruit and vegetables, whose jobs do not have a high level of education and who engage in little physical activity. This profile of heavy drinkers matches the results of earlier studies that have linked alcohol consumption and smoking with higher meat intake and lower intakes of fruit and vegetables (Padrao *et al.*, 2007, 2011). At the same time, individuals with this characteristic profile are predominantly carries of the allele 'A' of rs1800497 and of the allele 'G' of rs877138.

Limitations of the study, our study considered as 'cases' men who consume >70 g/day and women who consume >42 g/day. These cut-off values enabled a large number of excessive consumers to be included in the sample, but at the same time this fact might have contributed to a reduction in the power to detect association between SNPs and alcohol excessive consumption. Another limitation is that the EPIC project has not been designed to take dates about negative life events, stress and social support that have predictive utility (Farris *et al.*, 2015).

In summary, it is well known that candidate genes involved in dopaminergic pathway are of immense interest in a wide range of addictive disorders. Dopaminergic pathway gene polymorphisms have been extensively studied with respect to addictive disorders. However, no evidence indicating any strong association between alcohol dependence and polymorphisms of dopamine pathway genes has emerged from the literature (Bhaskar and Kumar, 2014).

In this sense, we have evaluated a wide panel of polymorphisms related to the dopaminergic pathway and detected statistically significant association for several SNPs in genes *DRD2* and *MAOA* with excessive alcohol consumption, while no associations were observed with *TH, SLC18A2, DRD1, DRD3* and *COMT*. Difference between the men and women with regard to the potential effects of the polymorphisms on excessive alcohol consumption is noteworthy, since rs10891556 in the *DRD2* gene proved to be the only SNP positively correlated with alcohol excessive consumption in both men and women. As discussed above, differences between the men and women emerged for the remaining SNPs associated in this gene. The main results of this study suggest that future research should not only include patients with significant alcohol dependence but should also seek to ensure that both sexes are suitably represented in these samples, bearing in mind the genetic and lifestyle variations attributable to the cited variable.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *Alcohol and Alcoholism* online.

FUNDING

This study has been funded partially by a grant from the Spanish Ministry of Health and Consumer Affairs (MSC07/DROG01). The authors thank for technical and human support provided by 'General genomics service sequencing and genotyping unit' of SGIker of UPV/EHU.

CONFLICT OF INTEREST STATEMENT

None declared.

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