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Original Article

Association of inflammatory gene variants with problematic alcohol use in a Colombian population

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Abstract

Alcohol dependence is a multifactorial disease that constitutes a significant public health concern and a significant risk for individual, family, and social health. Its genetic component exhibits significant ethnic variation and is closely associated with the personal evolution of the disease. However, although multiple loci have been identified, no functional variants have been identified. In this work, we selected some genes from the inflammatory response pathway and searched for SNV (single nucleotide variants) in their promoter region that could be associated with the disease. We compared cases of problematic alcohol consumption (n=66) with controls (n=73) in a population sample taken at the National University of Colombia, Bogotá headquarters. Peripheral blood DNA extraction was performed. We used PCR and Sanger sequencing to find 28 SNVs and one STR in 10 inflammatory response genes that are connected to alcoholism. Then, using various bioinformatic tools, the analysis of haplotypes, linkage disequilibrium, epistasis and genetic networks was carried out. Allele and genotypic frequencies for this Colombian population were reported for the first time. Additionally, we found haplotypes that could be protective and risk factors for the disease, and gene interactions that have cumulative effects related to the drinker phenotype. The investigation of haplotypes, gene interaction, and gene networks is a highly effective methodology for identifying potential associations in small samples. Additionally, SNCA, IL-6R1, TNFR1, and MIF genes were profiled for further studies.

Keywords: Inflammatory response, Single nucleotide variant (SNV), Haplotype, Epistasis, Alcohol.

1. Introduction

Inflammatory-based human diseases are well known: atherosclerosis, asthma, Crohn's disease, multiple sclerosis, rheumatoid arthritis, etc. Knowledge about the production and effect of proinflammatory cytokines in alcohol dependence and related phenotypes and their effects on various organs such as the brain, liver, muscles, etc. has increased in the last two decades. It is such a multifactorial psychiatric disease that seeing it as an inflammatory condition opens the possibility of looking for new diagnostic or follow-up biomarkers and new therapeutic agents to treat some of its harmful effects [1,2]. Specific variants of genes related to the inflammatory response have been associated with the risk or susceptibility to alcohol addiction and related phenotypes and could be excellent therapeutic targets [3].

It is known that prolonged and heavy alcohol consumption promotes the production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 1β (IL1β), interleukin 6 (IL-6), nitric oxide synthase (NOS), etc., and in general, the inflammatory response, contributing to liver fibrosis and cirrhosis, among others. The brain, which is perhaps the most affected by this type of consumption after the liver, is prone to an inflammatory response with persistent behavioral changes and neurodegeneration [4,5].

Alcohol can also have a profound impact on the brain via three distinct pathways. Firstly, a neural pathway known as the vagus nerve can be sensitive to various proinflammatory molecules, such as IL-1β and SNCA, thereby transmitting signals to the central nervous system. A second pathway is the humoral route, which encompasses the diffusion of cytokines through the blood-brain barrier in two distinct ways. Lastly, the cellular route is utilized to facilitate the entry of active cells, such as monocytes, into the brain, resulting in the production of a diverse array of cytokines that induce modifications in gene expression, including those pertaining to the cell cycle and apoptosis [6]. It can be determined at the serum level and may be a reflection of what happens in the brain [7].

In contrast, it has been observed that serum endotoxin levels increase due to increased intestinal permeability due to the effect of alcohol. As an illustration, the component of the intestinal biota known as lipopolysaccharide (LPS) has the ability to induce neuroimmunity genes associated with states of depression and anxiety, which are also indicative

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of alcohol dependence [8]. LPS increases the expression of TNF-α in the liver and blood for 24 to 48 hours, while in the brain it persists for at least 10 months. This leads to degeneration of dopaminergic neurons, dopamine hypofunction, and sensitization of the alcohol reward system. This plays an important role in the pathogenesis of alcohol use disorders [9]. Furthermore, alcohol contributes to the oxidative stress experienced by neurons by enhancing reactive oxygen species (ROS), decreasing antioxidants such as glutathione, and reducing the activity of enzymes such as glutathione peroxidase, catalase, and superoxide dismutase, thereby contributing to their neurodegeneration [10]. Toll-like receptors (TLR) and other cytokine receptors share signaling pathways in the brain that converge on NFkB and alter gene expression, such as miRNA, leading to epigenetic changes and the induction of neuroinflammation genes, which is a novel approach to treating alcohol abuse disorders [11, 12].

The study focuses on the genes: α-synuclein (SNCA), which plays a significant role in neuroprotection and neurotoxicity [13-15]. Once a protein is out of the nerve cell, it could misfold, and some of these forms could activate microglia, a process that can become chronic and make continued inflammation harmful to the patient's brain [16]. However, the Rep-1 variant has been associated with the SNCA promoter, a microsatellite comprising distinct alleles. The shorter allele of the gene is associated with a lower expression and a higher predisposition to desire alcohol consumption. Excessive alcohol consumption increases the risk of neurodegenerative diseases, especially in individuals who have easy-to-aggregate variants [17]. Furthermore, the 3′-UTR region of the α-synuclein gene may be prone to variants that alter the secondary structure of mRNA, alter miRNA binding, and, consequently, affect gene expression [18].

Interleukin 6 (IL-6) and its receptor 1 (IL-6R1) are pleiotropic immune response glycoproteins that possess significant functions at the hepatic level and, in general, at the multisystemic level [19, 20]. Several studies have revealed that polymorphisms in the promoter of the IL-6 gene exhibit favorable associations with alcohol consumption and an increase in its serum concentration. However, the IL-6 and IL-6R1 pathways have been found to be involved in the pathogenesis of severe depression and psychosis and may be new therapeutic targets in the treatment of these psychiatric and inflammatory conditions [21- 23]. It has been reported that alcohol users have altered IL-6 serum levels, either at baseline or during withdrawal [24].

Tumor Necrosis Factor α (TNF-α) and its receptors (TNFR) are attributed a central role in the regulation of the immune response, both innate and adaptive, and therefore in the inflammatory response. LPS is an important inducer of TNF-α, both in vitro and in vivo, which interacts in a complex way with the immune system and with the vascular system, leading to the release of other cytokines. TNF-α levels are higher in heavy and occasional drinkers than in teetotalers and usually are associated with liver problems [24-26].

Interleukin 12 (IL-12) is a proinflammatory cytokine that plays an important role in inducing immunological abnormalities found in alcohol dependence, regardless of whether or not alcoholic liver disease is present. During the initial phase of alcohol dependence, the interferon alpha (IFN-α) and interferon-gamma (IFN-γ) pathways are activated, increasing the sensitivity of liver cells to TNF-α and the production of IL-12 [27-29].

Nitric oxide (NO) is a small intracellular and intercellular signaling molecule that stimulates the formation of reactive nitrogen species and cellular toxicity [30]. The use of inhibitors of this enzyme, such as L-N6-(1-iminoethyl) lysine hydrochloride, has been an effective drug in preventing the manifestations of alcohol dependence [31].

The macrophage migration inhibitory factor (MIF) is a pleiotropic protein that regulates both innate and acquired immunity and individuals who consume excessive amounts of alcohol tend to increase its expression. Ibudilast is a MIF inhibitor drug that has good effects in patients with alcohol consumption problems and in animal models of alcohol dependence. It is recommended to evaluate MIF as a biomarker to predict the psychotherapeutic and pharmacological response in patients with alcohol consumption issues, rendering it a viable candidate for a therapeutic target [32].

The Activated B-cell kappa light chain-enhancing nuclear factor (NFkB) is a ubiquitous transcription factor that has numerous target genes and has the potential to influence numerous cellular processes. 12 SNVs have been found in both the promoter and coding regions associated with alcohol dependence that appear to affect the risk of alcohol dependence, contributing particularly to an earlier onset of the disease [33].

The high mobility group boxes 1 (HMGB1) protein plays a crucial role in DNA repair, autophagy, and specific physiological responses. According to studies conducted on hippocampal brain slices, alcohol exhibited an increase in the levels of HMGB1/IL-1 complexes in culture media. These studies suggest that this pathway is a new neuroimmune mechanism in alcohol dependence [34].

The objective of this work was to determine SNV in the promoter region of the previously described genes related to alcohol dependence and the inflammatory response pathway in a university population residing in Bogotá, Colombia. 10 candidate genes with 28 SNV and one length variant (STR) were included.

2. Materials and methods

2.1 Study population and sample size selection

A descriptive study of type, cases, and controls was conducted using non-probabilistic, exploratory sampling. The sample size was, for convenience, approved by the ethics committee of the Faculty of Medicine of the National University of Colombia. A solicitation was issued through the social media platforms of the University, located at the Bogota headquarters, to engage in the study, to which approximately 400 individuals responded. Over 200 individuals were selected according to the inclusion and exclusion criteria, of which 139 had a blood sample taken, 73 were classified as controls, and 66 were classified as cases (individuals with problematic alcohol consumption). Men and women with an age range between 18 and 61 years, born in Colombia, and residents of the city of Bogotá who were linked to the university, either as students, teachers, or administrative employees, and who agreed to participate by signing informed consent were included. The AUDIT (Alcohol Use Disorders Identification Test) questionnaire was applied to all the individuals included in the study as an instrument for classifying the severity of alcohol dependence, as validated in Colombia by Cam-

po and Ospina [35, 36]. The sample of individuals was selected for convenience, considering that they were of the same sex and similar ages as the cases and that they obtained AUDIT scores of less than seven. The selection of individuals with problematic use of alcohol was carried out considering the following criteria: individuals whose first problem with addictive substances was alcohol and who obtained AUDIT scores higher than seven. However, all of them, both controls and cases, underwent a semi-structured interview based on Scoppeta and Silla [37, 38], with the objective of obtaining additional details regarding their alcohol consumption habits, frequency, and quantity, medical status, personal and family history, and thereby enabling a clear distinction between the control groups and cases (Figure 1). One crucial factor in establishing the phenotype of interest was to accurately calculate the grams of alcohol consumed by the study participants. It is apparent that this measurement is very complex due to the diversity of existing alcoholic beverages, their different degrees of alcohol, and the various forms of presentation and consumption by participants. Considering these features, the grams of alcohol consumed on a typical day of alcohol consumption were calculated by utilizing the equation: grams of alcohol = volume (mL) x graduation x 0.8/100 [39]. The exclusion criteria were as follows: refusal to provide informed consent; presence of infections, allergies, ongoing pharmacological treatments, or liver or psychiatric diseases.

2.2. Selection and analysis of SNVs

For the selection of biomarkers, we searched the literature for the most frequently described proteins related to the phenomena of inflammation and alcohol dependence and then searched the NCBI databases for the sequence of their genes. The EPD database was utilized to identify the promoter region. The regions that were abundant in CpG islands were identified, and within these regions, SNV was sought. Previous research had demonstrated that SNV exhibited a correlation with phenotypes of elevated alcohol consumption, addictions, or certain psychiatric illnesses with a dual presentation of alcohol dependence (depression and anxiety). After establishing the polymorphisms to be determined, the primers were designed using the Primer3 or Primer-BLAST tools (Table S1).

PCR reactions were performed according to the recommendations of the practical PCR guides [40]. The DNA was added to each PCR tube with the respective master mix, ranging from 50 to 100 ng, although the assay can be performed with a minimum of 5 ng of DNA. Then the samples were transferred to the end-point thermocycler [41]. The hybridization temperature of the primers, specific for each variant, was determined pre-experimentally by temperature gradients (Table S1). The amplification was verified on 2% agarose gels stained with SYBR-safe dye (ThermoFisher, CN S33102) whose detection limit is 60 pg dsDNA per band. The SNV of the purified amplification was determined by DNA sequencing employing the Sanger method (Big DyeTerminator Cycle Sequencing Ready Reaction Kit, ThermoFisher, CN 4337455). The quantity of PCR product utilized was a minimum of 15 ng of DNA, although this quantity is contingent upon the size of the amplified DNA segment.

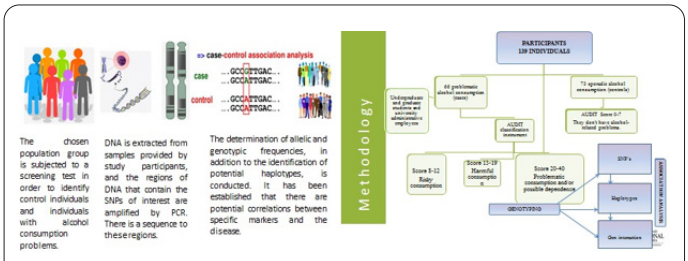

Fig. 1. General outline of carrying out an exploratory association study (A) and the step-by-step of the particular experimental methodology of this study (B). Own authorship.

2.3. Study of the STR-rep-1 of the SNCA gene

In the case of the long genetic variant of the SNCA gene called rep-1, the conditions for mounting and running conventional PCR had been described before [42]. Then, 1.5 uL of the amplified DNA was taken in 12 uL of formamide (MilliporeSigma CN 344206-M) and mounted in capillary electrophoresis. A volume of 0.5 uL of ROX-500 (ThermoFisher, CN 401734) was employed as a marker for molecular size. To perform capillary electrophoresis, the ABI PRISM 310 equipment was used with the POP-4 polymer (ThermoFisher CN 4393710), together with the genetic analyzer 1X running buffer (ThermoFisher CN 4335613) registered trademarks. Gene Marker Version 2.6 was used to identify the different alleles.

2.4. Statistic analysis

The continuous variables were expressed as the average +/- the standard deviation, while the categorical variables were expressed as a percentage. The IBM SPSS statistical program for Windows version 23 [43] was used to make these estimates. The genotypic and allelic frequencies of the SNVs were determined by direct counting and expressed as a percentage per group. The Chi-square test was used to compare these categorical variables and evaluate the Hardy-Weinberg genetic balance, for which the GENALEX 6 program was used. In this case, the statistical significance was estimated using Fisher's exact test and when the frequencies were very low, the Yates correction was used. The odds ratio (OR) and 95% confidence intervals (CI) were estimated to determine the association of each polymorphism with problematic alcohol use, taking the most frequent homozygous genotype as reference. In addition, inheritance models, haplotype study and linkage disequilibrium were analyzed using the programs: SNPStats and haploview. The epistasis analysis was performed with the help of the multifactorial dimensional reduction (MDR) program version 3.0.2, the promoter-transcription factor interaction and gene networks, using the Lasagna 2.0 and IMP (Integrative Multi-species Prediction) programs respectively. For all statistical analyses, a significance level of $p < 0.05$ was considered.

3. Results

3.1. Sociodemographic characteristics

The study included 139 individuals, of which 73 were classified as controls and 66 as cases. There were 39 women and 34 men in the control group, while there were 26 women and 40 men in the problematic alcohol usage group. The age range observed in both groups was comparable, with subjects ranging from 18 to 61 years old, with an average age of 27 and 29 years for both controls and cases, respectively. The majority of the participants in the

study were young students enrolled in undergraduate programs. Most people in the study started drinking alcohol before they turned 18, which is the legal drinking age for alcoholic beverages in the country. The control group was comprised predominantly of graduate students and professionals employed by the university, whereas the case group was predominantly comprised of undergraduates (Table 1).

The 73 individuals in the control group had an AUDIT score below seven, while the 66 individuals with problematic alcohol use had a score greater than seven. From this last group, 12% scored between 8 and 12, 35% between 13 and 19, and 53% between 20 and 40 on the AUDIT. More than half of these individuals could be classified as having possible alcohol dependence. The decision was made to include in the group of cases all scores greater than seven, which would at least be people with problematic alcohol use.

In order to better characterize and select the sample, a semi-structured questionnaire was additionally applied to the participants, which asked, among other questions, the average consumption of alcoholic beverages during a consumption session closest to the date of sampling. According to the participants, this reflected their average consumption per event. Approximately 50% of the controls consumed mainly beer, while the cases preferred to drink three different types of alcoholic beverages: beer, rum, and brandy. It was determined that the individuals in the case group consumed an average of 309±110 g of alcohol, whereas the control group consumed an average of 66±60 g per consumption event. If we were to examine the consumption session by gender, it was observed that the women of the control group consumed an average of 58 g of alcohol, whereas the men consumed 74 g. In contrast, the women of the cases consumed an average of 336 g and the men consumed 291 g of alcohol. The AUDIT score and the grams of alcohol consumed in a drinking session allowed us to clearly categorize our sample into control subjects and those with alcohol consumption problems, who matched 100%. Regarding the socioeconomic level, we did not find any differences between the two groups, presenting the same proportion of individuals in average condition as the majority of individuals. Approximately 60% of the individuals in the case group reported the consumption of other types of psychoactive substances, such as marijuana and tobacco, a significant difference compared to the control group. Also, it is noteworthy that more than 50% of the cases stated that they did not know if a family member had alcohol consumption problems (Table 1).

3.2. Genotypic and allelic frequencies of SNV in the promoter region of the selected genes

There were 29 genetic variants evaluated, but not all were successfully genotyped. We selected results with clear sequence electropherograms in both directions

Table 1. Sociodemographic characteristics of the study participants.

Parameter		Problematic Alcohol Consumption n (%)	Controls n (%)	Problematic Alcohol Consumption and controls (p-Value)
Age: mean ±SD (years)		29,7 ±10,4	27,2±7,6	0.060#
Range(years)		18-61	18-61	
Sex	Men	40 (61)	34 (47)	0.126
	Women	26 (39)	39 (53)	
AUDIT Score				
Range 0-7		0 (0)	73 (100)	0.000*
Range > 7		66 (100)	0 (0)	
Alcohol consumption (g, grams)		309,4±113,5	65,5±56,2	0.000*
Age of onset of alcohol use (years)$				
<18		54(82)	59(81)	0.482
>18		12(18)	9(12)	
Family members with consumption problems				
Yes		14(21)	28(38)	0.000*
No		5(8)	25(34)	
Do not know not answer		47(71)	20(28)	
Consumption of other psychoactive substances				
Yes		38(58)	15(21)	0.000*
No		7(11)	35(48)	
Do not know not answer		21(31)	23(31)	
Socioeconomic level				
Low		12(18)	9(12)	0.166
Half		16(24)	30(41)	
High		8(12)	7(10)	
Do not know not answer		30(46)	27(37)	

n= number of individuals, $Five control individuals have never consumed alcoholic beverages, *Statistically significant differences, Fischer's exact test p<0.005, # Analysis of variance p<0.005.

that did not generate uncertainty in their allelic assignment. Nine markers in four genes showed a single genotype in both groups, that is, they were monomorphic: SNCA (rs989496677, rs927159023, rs950036657, and rs916862395); TNFR1 (rs1030920578 and rs1032563104); NFkB-RELA (rs1039347100 and rs1394889070); and HMGB1 (rs41477046) and therefore do not present any utility in this study. Also, in all the genotypes of the variants analyzed, no statistically significant distinctions were observed. This implies that the genotype frequencies did not differ in the alcohol-dependent compared to the controls. However, in the allelic frequencies, an allelic pair rs1470654147 (C/T) of the IL-6R1 gene was observed, exhibiting statistically significant distinctions, where T was the minor allele (Table S2).

Similarly, when comparing the results of the only STR analyzed, rep-1, no statistically significant differences were found (Table 2). Eleven different genotypes were observed combining the eight alleles of different lengths determined for the sample taken. The alleles 269 and 271 bp were predominant for both groups, and the majority genotype for both groups was the heterozygote 269/271.

According to the frequencies of the alleles necessary to modify the risk, four models of inheritance were defined: codominant, dominant, recessive, and additive. Following these models and using the most frequent homozygous genotype as a reference, the association between the gene variants and susceptibility to alcohol dependence was evaluated, estimating the odds ratio (OR). None of them showed any association with alcohol dependence (Table S3). A logistic regression model was used to adjust the codominant model (the most general one, Table S4) and it was also adjusted according to the variables of confounding factors, such as sex, age, and age of initiation of consumption. An association was found between the T/T genotype of the rs4149621 marker of the TNFR1 gene and protection against alcohol dependence (Table S5).

3.3. Haplotype study

Using the SNPstats and haploview programs, the possible haplotypes for four groups of markers located in the TNFR1, SNCA, IFNGR1, and IL-12RB genes were analyzed. The haplotype frequencies and odds ratios of the participants were estimated (Table 3), after being adjusted for confounding variables such as gender, age, and age of initiation of consumption (Table S6).

Based on an LD > 90%, 5 haplotype blocks were defined (Figure 2): Block 1, TNFR1 (1rs2234649, rs4149621 and rs4149570); Block 2, SNCA (rs2619363, 2301134 and rs924048579); block 3, SNCA (-2171 and rs542037441); block 4, INFGR1 (rs7749390 and rs2234711) and block 5, IFNGR1 (rs17181457 and rs121913171). By incorporating those monomorphic markers and reducing the LD value, a variety of blocks per gene were constructed. Of these, only the -GGCAGCAGG- haplotype in the SNCA gene displayed haplotype frequencies in both controls and cases, with statistically significant differences. This suggests that this could be a potential protective factor against problematic alcohol consumption. Other markers with significant differences presented frequencies of zero or very close to zero, generating a very high risk or protection value, which could be a bias related to the small size of the sample. During the analysis, a haplotype in the TNFR1-GGTCT gene was identified in males, which was associated with protection against alcohol dependence. This haplotype was found to be associated with a frequency of 72% in the control group, with an OR of 0.08 and a confidence interval of 0.01-0.54 (Table S8). Also, in men, the -GGGA- haplotype in IFNGR1 was associated with risk with a very high OR. Perhaps this is due to the size of the sample (Table S6).

The linkage disequilibrium analysis of marker pairs was performed, obtaining the D, D´, and R statistics. Five pairs of alleles were observed to be in linkage disequilibrium, three of which presented high values for the D´ parameter, located in the SNCA and IFNGR1 genes (Figure 2 and Table S7). Analyzing pairs of markers, the IFNGR1 gene haplotype between markers rs121913171 and rs2234711 presented a strong linkage disequilibrium D´= 0.9 and its associated phenotype. The aforementioned gene exhibited a second haplotype encompassing the markers rs2234711 and rs7749390, exhibiting a significant imbalance of D´>0.8, and the CA variant was associated with protection. Similarly, biallelic haplotypes were identified with the markers rs4149621 and rs2234649 in the TNFR1 gene. The AC haplotype is associated with protection, despite its D´=0.48 indicating a moderate linkage disequilibrium. Lastly, in men, the markers rs2619363 and rs2301134 of the SNCA gene revealed the CT haplotype associated with protection against alcohol dependence, exhibiting a strong linkage disequilibrium (D´=0.7).

3.4. Gene interaction

Another type of genetic interaction, epistasis, could explain a large percentage of the variability in alcohol dependence and related phenotypes. In our analysis, we used the MDR method, which was designed for case-control association studies with a small sample size. Twenty polymorphic SNVs (40 alleles) of the selected genes were used, and the selected environmental type characteristics were: sex, age, and age of onset of alcohol consumption. The total number of attributes was 23. With the MDR, we evaluated all the interactions of two and three attributes, including both genetic and environmental variables, adjusted for confounding variables (Figures 3 and 1S). Pie charts based on entropy and the dendrogram were obtained, illustrating the interaction between the genetic and environmental variants. The red line indicates a

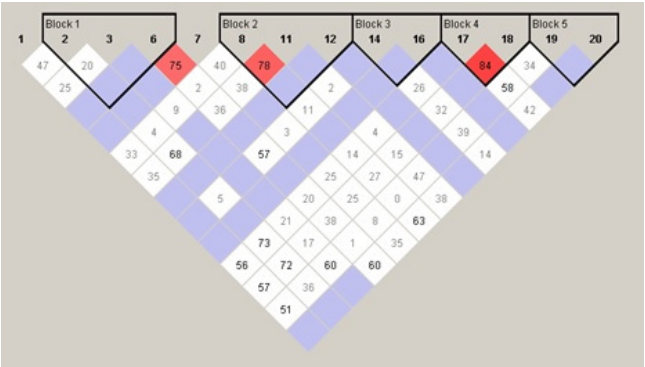

Fig. 2. Haplotype block scheme. The numbers inside the diamonds are D' values for the respective SNV pairs. Solid red diamonds represent high LD with high significance, blue diamonds represent strong LD with low significance. Numbers in gray within white diamonds represent evidence of recombination. Blocks of haplotypes determined using the Haploview software are depicted.

Table 2. Genotypic and allelic frequencies of STR Rep-1 of the SNCA gene in controls and individuals with problematic alcohol consumption.

Genotypes Rep-1 SNCA	Genotypic frequency n (%)		Alleles Rep-1 SNCA	Allele frequencies n (%)	
	Controls n=46	Problematic use of alcohol n=49		Controls n=46	Problematic use of alcohol n=49
263/263	1 (2,2)	0 (0,0)	**263**	2 (2,2)	0 (0,0)
265/269	0 (0,0)	1 (2,0)	**265**	0 (0,0)	1 (1,0)
267/267	0 (0,0)	1 (2,0)	**267**	1 (1,1)	10 (10,2)
267/269	1 (2,2)	8 (16,3)	**269**	37 (40,2)	37 (37,8)
269/269	8 (17,4)	7 (14,3)	**271**	49 (53,3)	44 (44,9)
269/271	19 (41,3)	14 (28,6)	**273**	3 (3,3)	3 (3,1)
269/273	1 (2,2)	0 (0,0)	**275**	0 (0,0)	2 (2,0)
271/271	14 (30,4)	13 (26,5)	**277**	0 (0,0)	1 (1,0)
271/273	2 (4,3)	3 (6,1)	**X^2 (p-value)**	**13,456 (0,062)**	
271/275	0 (0,0)	1 (2,0)			
275/277	0 (0,0)	1 (2,0)			
χ^2 (p-value)	**12,423 (0,333)**				

Table 3. Frequencies of haplotypes of markers and association with risk of susceptibility to problematic alcohol consumption in cases and controls, adjusted for sex, age, and age of onset of consumption.

Gene	SNV´s	Haplotypes	Freq control	Freq cases	Odds ratio	(95% CI)	P Value
TNFR1	rs1030920578 rs1032563104 rs4149621 rs4149570 rs2234649	GGTCT	0,79	0,65	1	----	---
		GGCCT	0,14	0,11	0,66	0,15-2,80	0,57
		GGTAT	0	0,14	3,08	0.15-61.8	0,47
		GGCAG	0,056	0,024	0,87	0,12 -6,16	0,89
		GGTCG	**0**	**0,057**	**$4,7X10^{21}$**	**4,7-4,7(10^{21})**	**<0,000***
		GGCCG	**0**	**0,024**	**$7,1X10^{8}$**	**7,1-7,1(10^{8})**	**<0,000***
SNCA	rs2619363 rs542037441 rs989496677 -2171 rs927159023 rs924048579 rs2301134 rs950036657 rs916862395	GGCTGCGGG	0,31	0,45			
		GGCTGCAGG	0,20	0,20			
		TGCTGCAGG	0,17	0,10	1	--	--
		GGCAGCGGG	0,13	0,11	0.51	0,12 –2,22	0,37
		GGCAGCAGG	**0,10**	**0,06**	0,28	0,05 –1,59	0,15
		TGCAGCAGG	0,03	0,08	0,11	0,01-1,51	0,1
		TGCAGCGGG	0,05	0	**0,15**	**0,03-0,83**	**0,032***
		GTCTGCGGG	0	0,009	0,92	0,08-10,85	0,95
		TGCTGTAGG	0,001	0			
		TGCTGCGGG	0	0			
		TGCTGTGGG	0	0			
IFNGR1	rs121913171 rs17181457 rs2234711 rs7749390	GGAA	0,57	0,57			
		GGGG	0.24	0,26			
		GAGG	0,10	0,04	1	---	--
		GAAA	0,05	0,02	1,17	0,46 -3,01	0,75
		GGGA	**0**	**0,05**	0,45	0,10 -1,97	0,29
		GGAG	0,02	0,03	0,57	0,08- 4,09	0,58
		AGGG	0,01	0,01	**$1,8X10^{7}$**	**$1,8X10^{7}$-$1,8X10^{7}$**	**<0,000***
		AGGA	0	0,01	2,40	0.19 - 30.63	0,5
		GAAG	0	0,01	0,57	0,04 – 7,49	0,67
		AGAG	0,01	0			
IL12RB1	rs436857 rs393548	GT	0,82	0,90	1	--	--
		AA	0,08	0,05	0,51	0,12 – 2,27	0,38
		GA	0,06	0,05	0,48	0,08 – 2,88	0,43
		AT	0,03	0	0,00		1

*Statistically significant differences with P<0.05.

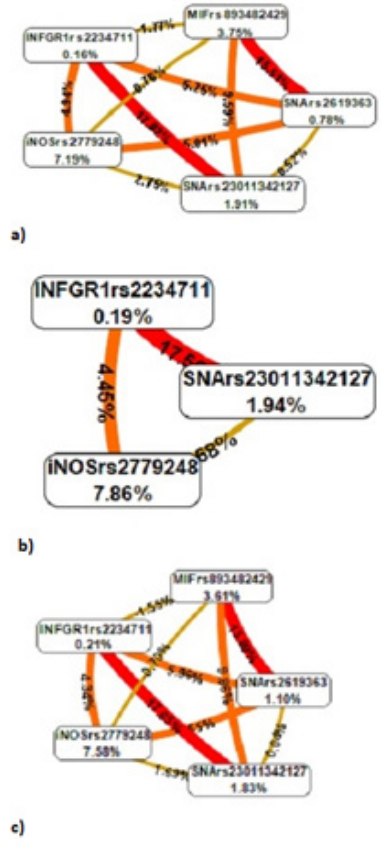

Fig. 3. Entropy-based circle graph of gen-gen interactions in a population of individuals with problematic alcohol use adjusted for sex (a), age (b) and age of onset of alcohol consumption (c).

strong synergistic interaction effect between the SNCA (rs2301134) and IFNGR1 (rs2234711) markers in all three models presented; similarly, the SNCA (rs2619663) and MIF (rs89348249) markers indicate a strong interaction in the model adjusted for gender and age of consumption initiation.

To determine the biological significance of our findings, we used the IMP bioinformatics tool in which the genes of interest were introduced, which interact according to our findings. A list of biological processes predicted for the genes in question and a network with genes predicted to be functionally related to them were obtained. The identified interconnected genes analyzed in this study were involved in the regulatory and production pathways for cytokines and neurotransmitters (Figure 2S).

4. Discussion

The first study in Colombia is presented, in which 29 gene variants in 10 inflammatory response genes were evaluated in a sample of individuals with problematic alcohol consumption compared to a control group. The control group had a balanced proportion of men and women, while the alcohol consumers had a higher proportion of men, almost double that of women. One of the characteristics of alcohol dependence in women is its secrecy, perhaps due to fear of the social perception towards consumption in women, which is different from that generated in men [44]. Both the controls and the cases showed that more than 80% of the participants started drinking alcohol before they were 18 years old. In the group of problematic alcohol consumption, students from undergraduate academic programs predominated, which confirms the trend observed worldwide of greater alcohol consumption in adolescents and children. This behavior has been erroneously trivialized and naturalized [45, 46]. However, the average amount of alcohol consumed in grams per event by women in the case group was higher than in men, a phenomenon that has also been observed in other countries recently [45]. A statistically significant difference was observed in the consumption of other psychoactive substances by the group of cases compared to the controls. In the case group, participants were classified as poly-drinkers because it was almost impossible to find only alcohol users. All the data collected in recent years indicate that the consumption of psychoactive substances in multiple quantities is the most prevalent behavior worldwide, and alcohol is found in 90% of the poly-consumption among diverse populations [47].

The disease of alcohol dependence is multifactorial, and genetic and environmental variants can exert an impact on susceptibility both individually and in concert [48, 49]. Similarly, in the controls, the rs2850015 marker of the IFNAR1 and, in both cases and controls, the rep-1 and rs4149621 markers of the SNCA gene were found to be in disequilibrium (Table S5). It is possible that the small sample size generated random changes in the allelic and genotypic frequencies, and that they are not representative. However, it is possible that there were errors in the genotyping, which we tried to minimize at the time of experimental development (sequencing in both directions, taking only clean and well-defined sequences) and performing a careful analysis. The frequencies obtained in this study were compared with those reported worldwide for the European, African, and Latin American populations, considering the ethnicity of the Colombian population. The website of the alpha project (allelic frequency aggregator) was consulted [50]. Upon comparing these data (Table S7), it was evident that the majority of the allelic frequencies obtained were remarkably similar to those reported for Europe. Some closely resembled those reported for Latin America, and a few were remarkably similar to those reported for Africa. In Bogotá, the sample was taken and the result shows that its inhabitants are of different ethnic backgrounds. This has been seen in other types of genetic markers [51]. Based on the aforementioned information and HWs equilibrium analysis, it can be inferred that the sample was adequate and representative for the purpose of conducting our analyses. As a noteworthy finding, it was observed that the frequencies of three markers identified in this study, namely SNCA-2171, SNCA-rs9224048579, and TNFR1-rs1032563104, were not reported in any other populations worldwide. Moreover, the marker SNCA -2171 was a polymorphic variant that was first reported by our research group in previous studies associated with this line of research, and it was confirmed in this study [52].

In this study, it was determined that the T allele of rs1470654147 of the IL-6R gene was solely associated with a greater susceptibility to problematic alcohol consumption for its carriers. However, no differences were observed in the synthesis of IL-6R mRNA and protein when comparing the two groups without stratifying, despite the presence of differences stratifying by sex of the participants [42]. This variant is situated at position -423, upstream of the translation start site. Using the Lasagna 2.0 program [53], a thorough search was conducted to identify the transcription factors that could potentially

interact with this region of the promoter. The results revealed that SP1 and HNF-4 are the two factors that bind to delimited sequences within the polymorphism. Particular interest is the factor SP1, a ubiquitous transcription factor that regulates the transcription of housekeeping genes [54]. In certain populations, a correlation between various polymorphisms in IL-6 genes and other interleukins and their receptors and psychiatric diseases has been observed, indicating their significance during early development and their impact on neurodevelopment and susceptibility to mental illness. However, this association has not been observed with alcohol dependence or similar phenotypes [55].

Some authors have conducted association studies of SNVs in the SNCA gene and alcohol dependence in patients of European origin, without finding a relationship with dependence. However, they did find a relationship between intense alcohol seeking with eight SNVs in this gene [33]. Furthermore, they had associated an intermediate phenotype such as alcohol tasting with two SNVs (rs2583985 and rs356168) of SNCA and brain-generated responses [56]. Only one length polymorphism in the SNCA gene, called Rep-1, had been associated with susceptibility to alcohol dependence, with the short allele being more frequent in individuals with problematic alcohol use. This variant results in lower SNCA expression in the brains of alcohol-dependent patients [14, 57]. In our study, no significant differences were found between the groups for this Rep-1 length variant, but the short allele (267 bp) was 10% more common in people with problematic alcohol use and the P-value was close to the statistically significant difference. This suggests that it is necessary to increase the sample size to confirm or refute this finding.

When stratifying the sample by sex, it was found that the AA genotype of the rs4149621 marker of the TNFR1 gene in men was associated with protection against susceptibility to alcohol dependence. It is widely acknowledged that prolonged alcohol consumption leads to inflammation at the intestinal level, resulting in an elevation of TNF-α levels and a consequent increase in intestinal permeability, ultimately leading to liver inflammation. The interaction between TNF-α and TNFR1/2 is essential for signal transduction, and pharmacological intervention on this pathway has shown that the stabilization of the intestinal barrier decreases inflammation and protects against the action of alcohol. Perhaps polymorphic variants such as the one found in this study would alter this signaling pathway and the inflammatory response of the organism in men [58].

In most multifactorial pathologies, the genetic factor is due to the coherence of multiple low-risk variants that have synergistic effects, which means that their effect is greater than the individual sum of the effect of each variant separately. Furthermore, the threshold effect is presented, which is a factor addition limit below which the trait is not expressed. Furthermore, racial affiliations play a significant part in its variation. For a pathology such as alcohol dependence, it is critical to discover biomarkers for the detection of susceptibility. Therefore, it is necessary to analyze multiple SNVs in different genes and populations with large samples.

Given that the contribution of individual markers was diminished, particularly in a sample of such limited size, we conducted an analysis of several inherited SNV en block within the same gene, namely the haplotypes. Our analysis focused on four genes, namely TNFR1, SNCA, IFNGR1 and IL-12RB1 (Tables S8 and S9). Four haplotypes were associated with susceptibility, three of which had null or very low haplotype frequencies in one of the groups, which could be related to the size of the sample and present limitations at the time of analysis. This was the case for the IFNGR1 gene, where the GGGA haplotype in men with problematic alcohol consumption was associated with susceptibility. A haplotype called GGCAG-CAGG was found in the SNCA gene that was associated with protection. On the contrary, after adjusting the data for covariates such as gender, age, and age of initiation of consumption, it was discovered that the GGTCT haplotype was present in the TNFR1 gene in males, which is also associated with protection. In the literature, there exist numerous studies examining the association between haplotypes and alcohol dependence, wherein associations between the disease and a specific haplotype have been reported in genes such as galanin, dopamine receptor 2 (DRD2), certain taste receptors such as TAS2Rs, GABA neurotransmitter receptors (GABRG1 and GABRA2), corticotropin-releasing hormone receptor (CRHR1), a monoamine transporter (SLC18A2), among others. Only haplotypes for the alpha-synuclein gene (SNCA) and its poor association with alcohol dependence or similar phenotypes have been reported [59-63]. The haplotype identified in this study in male cases located in the promoter of the TNFR1-GGTCT gene was associated with protection against problematic alcohol consumption and its lower protein expression [42]. This may be attributed to a decrease in the affinity of transcription factors, resulting in a reduction in the rate of transcription and translation.

The MDR analysis indicated that combining these pairs of markers, namely SNCA (rs2301134) and IFNGR1 (rs2234711), SNCA (rs2619363) and MIF (rs893482429), into a single variable yielded additional information compared to merely considering them in a linear or additive model. The OR values of 20 and 13 respectively, along with a sufficient confidence interval and significant P-values, were represented in the graphs with red lines. A moderate interaction between the markers (SNCA rs230113 and iNOS rs2779248) and (IFNGR1 rs121913171) was shown in the yellow lines. With these results, we considered that these three genes: SNCA, IFNGR1 and MIF were an interesting target for further studies.

The MDR found that the interaction between the SNVs of the genes SNCA-rs2301134, iNOS-rs2779248 and IFNGR1-rs2234711 was the best prediction model. There were differences in genes SNCA-rs2619363, MIF-rs893482429, and SNCA-rs2301134, iNOS-rs2779248, and INFGR1-rs2234711. The model was capable of predicting these disparities with a precision of 69%, 67%, and 71%, and a valid cross of 6/10, 7/10, and 7/10, with a p-value of 0.0001 (Table S10). Out of the resulting network of genes, the STAT proteins stand out, which were associated with receptors that, when activated, phosphorylate them, translocate to the nucleus, and act as transcription factors. The INF (α and γ), and IL-6 signaling pathways can be involved in the activation of STAT 1 [64]. Furthermore, the MAPT proteins stand out, Tau molecules are associated with the assembly of microtubules in neurons and are essential in the maintenance of the structure of these cells and the transport of materials [65].

Based on these results and previous results of expres-

sion [42]and methylation [66], the TNFR1, IL-6R, SNCA, and IFNGR1 genes are emerging as candidates for biomarkers of the disease with their respective molecular modeling [67].

This exploratory study provides new insights into the genetic factors underlying problematic alcohol consumption in the Colombian population. By analyzing single nucleotide variants (SNVs) and haplotypes in key inflammatory response genes, we identified novel genetic profiles that may influence susceptibility or protection against alcohol dependence. Our findings highlight the potential of genes such as TNFR1, IL-6R, SNCA, and IFNGR1 as promising biomarkers for alcohol-related disorders. These results underscore the importance of considering genetic and inflammatory pathways in the assessment and management of alcohol use disorders. Further research with larger and more diverse populations is warranted to validate these associations and to explore their potential for clinical application in personalized prevention and treatment strategies.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Consent for publications

The authors have read and approved the final manuscript for publication.

Ethics approval and consent to participate

The ethics committee at the National University in Colombia approved this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

MR, FA: Conceptualization, methodology. MR: Investigation, software, statistical analysis, writing original draft preparation. FA: Reviewing and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

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