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Inflammatory response genes differentially expressed in a Colombian university cohort with alcohol consumption problems

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ABSTRACT. Alcohol dependence is a multifactorial inherited psychiatric disease that is difficult to diagnose and treat. Recent years have seen an increase in knowledge about molecular pathways and effect of proinflammatory cytokines on alcohol dependence. Understanding this pathology as an inflammatory condition opens the possibility of using new therapeutic agents to treat its harmful effects. The objective of this study was to determine if there was a difference in gene expression of inflammatory response genes between two groups of university Colombians, one with a problem with alcohol (n=25) and one without, as a control (n=25). In previous research conducted by our research group, involving more than 30 single nucleotide variants of more than 10 inflammatory response genes, certain haplotypes, interactions, and gene networks were identified that indicated differences between alcoholics and controls in the genes SNCA, Il6R1, TNFR1, and MIF. Total RNA from blood mononuclear cells was extracted by determining the relative expression by qPCR of these genes; using ELISA, the concentration of their protein products in plasma was determined. There were differences in the relative expression of the TNFR1 and MIF genes,

with a decrease in individuals with problematic alcohol consumption. The relative transcription of SNCA increased in men, whereas there were no changes in women. Nevertheless, there were no significant differences observed in the SNCA, IL6R1, and TNFR1 proteins, whereas MIF was decreased in the overall sample. On the other hand, SNCA, IL6R1 and MIF proteins showed differences in men. We observed disparities in the expression of these inflammatory response genes when comparing controls and cases. These findings demonstrate that SNCA, IL6R1, and MIF are candidates for further study as potential therapeutic targets in disease conditions related to alcohol abuse.

Key words: Inflammatory response; Gene expression; Therapeutic target; Alcohol; mRNA; Protein

INTRODUCTION

Alcohol dependence is a psychiatric disease that is complicated, chronic, progressive, and sometimes fatal. In the last few decades, it has become one of the biggest public health problems in relation to legally used psychoactive drugs. It has been linked to more than 200 diseases, such as cancer, stroke, liver cirrhosis , psychiatric diseases, etc. The World Health Organization attributes 2.8 million annual deaths worldwide due to alcohol dependence, which corresponds to 2.2% of deaths among women and 6.8% among men (Organización Mundial de la Salud, 2005). As in the rest of the world, alcohol is an important psychoactive substance in Colombia. Nearly seven million people between the ages of 12 and 65 are alcohol consumers, which is equivalent to 35% of the population in that country's age range. Around 2.4 million people have risky or harmful habits, which is 35% of all consumers and 12.5% of the total population in this age range (Misterio de salud y protección social, 2013). Alcohol dependence can be difficult to diagnose and treat, and one of the most promising approaches involves molecular genetics.

Studies with candidate genes and particularly single nucleotide variants (SNVs) trials in humans have not been sufficient to explain the complex mechanisms of the neurotoxic effect of alcohol and the various phenotypes of alcohol dependence. Even though the panorama is complex, studies of total genomics, transcriptomics and proteomics show us that there are some networks closely linked to the functional changes that occur. On the other hand, assays to determine the effect of alcohol on laboratory animals show changes in the expression and function of various genes and/or proteins. Similar results have been obtained in humans (Singh et al., 2007; Tabakoff et al., 2009). An addiction can last a lifetime and even return after many years of abstinence. This suggests that excessive alcohol consumption can cause long-lasting alterations in the brain that last for years. Those changes are similar to changes that psychoactive drugs like alcohol cause, such as changes in the chromatin or DNA structure, and the induction or repression of non-coding RNA (Robison and Nestler, 2011; Vagga et al., 2021).

Despite the differences in their mechanism of action, virtually all psychoactive substances increase dopaminergic activity from the ventral segmental area to the nucleus accumbens. Long-term consumption of alcoholic beverages can affect the expression of

numerous genes in the brain. One of the main transcription factors affected is the delta member of the Fos family of transcription factors, FOSB (Li et al., 2010). Furthermore, changes in NFkB (nuclear factor kappa light chain enhancer of activated B cells) and MEF2 play an important role in response to stress and are affected by the action of ethanol(Chen et al., 2017; Nennig and Schank, 2017). Also, CREB, a transcription factor that is triggered by psychoactive substances, is deregulated (Wand, 2005). In addition to the previously mentioned metabolism and reward system, signal transduction genes (MAPK1, NTRK2, SOD2), and inflammation genes (TNFR1, IL-1R, IL-6R, TLR2, TLR4 and NFkB) are also involved (Sohma et al., 1999; Tabakoff et al., 2009; Levey et al., 2014).

In the last two decades, knowledge about certain molecular pathways has increased, such as the production and effect of proinflammatory cytokines and the production of reactive oxygen species (ROS) in alcohol dependence and its effects on various organs, such as bones, muscles, and brain. Seeing alcohol dependence as an inflammatory condition opens the possibility of using new therapeutic agents or agents used to treat other diseases related to inflammation to treat some of the negative effects of this disease (González-Reimers, 2014).

It is known that alcohol changes the levels of cytokines in various tissues and organs. These changes contribute to liver diseases such as cirrhosis and fibrosis. They affect the brain with long-term changes in behavior and neurodegeneration (Crews et al., 2017). Furthermore, the immune response of the peripheral system contributes to the production of cytokines and chemokines, such as monocyte chemotactic protein (MCP), tumor necrosis factor alpha (TNF-α), interleukin 1β (IL1β), interleukin 6 (IL6), Toll-like receptors (TLR's) and high mobility group proteins (HMGB1) that are increased with chronic alcohol consumption. NFkB is one of the targets of these signaling molecules. As a result, it increases the activity of enzymes such as NADPH oxidases (NOX), cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS). Like stress, alcohol can change the immune response genes (Crews and Vetreno, 2016).

We selected some of the genes for this study from the results of a previous study on SNVs in the promoter region, haplotypes and epistasis (Rey M and Aristizabal FA, results not yet published). Alpha synuclein (SNCA) is important in neuroprotection and neurotoxicity processes, and its absence increases the sensitivity of the brain reward system in chronic alcohol consumers (Janeczek and Lewohl, 2013; Tesoriere et al., 2014; Ziolkowska et al., 2008). However, SNCA could fold in the wrong way once outside the nerve cell, and some of these forms could activate microglia, the main defense mechanism in the central nervous system (Caplan and Maguire-Zeiss, 2018). This process could become chronic and turn into a disease. On the other hand, the SNCA promoter has been mostly linked to the Rep-1 variant, a dinucleotide repeat polymorphism with different alleles. In some populations, it has been found that the allele is shorter, the lower the gene expression, and the greater the predisposition to desire to consume alcohol. The risk of neurodegenerative diseases increases with excessive alcohol consumption, especially in individuals who have easy aggregation variants, especially with certain SNVs in the coding region of the gene (Janeczek et al., 2014; Janeczek and Lewohl, 2013). Furthermore, it has been suggested that polymorphisms in the 3-UTR region of the alpha-synuclein gene may be associated with modifications in the secondary structure of mRNA and the binding of miRNAs to it, thereby influencing gene expression (Nunez and Mayfield, 2012)

The immune response glycoproteins Interleukin 6 (IL6) and its receptor 1 (IL6R1) have important functions at the hepatic level and in general at the multisystemic level (Nguyen et al., 2017; Tanaka et al., 2014). Some studies have found that polymorphisms in the IL-6 gene promoter are positively associated with alcohol consumption and IL-6 concentrations. On the other hand, other studies have found that the IL-6/IL6R1 pathways are involved in the pathogenesis of severe depression and psychosis, and may be new therapeutic targets in the treatment of these psychiatric and inflammatory diseases (Carnota, 2002; Kashem et al., 2020; Mukamal et al., 2007). The levels of IL6 have been reported to be altered in alcohol consumers, either at the beginning or during withdrawal (Heberlein et al., 2014). This pathway is interesting for the search for therapeutic targets for alcohol dependence.

Tumor necrosis factor alpha (TNF-α) and its receptors (TNFR) are thought to play a big part in the regulation of the immune response, both innate and adaptive, and therefore in the inflammatory response. The lipopolysaccharide (LPS) increases during alcohol dependence and is a good inducer of TNF-α, which works in a complex way with the immune system and the vascular system. This results in the release of other cytokines and the expression of new ones such as PAF (platelet activating factor) and eicosanoids (Organización Mundial de la Salud, 2005; Emamzadeh, 2016). Heavy and occasional drinkers have higher levels of TNF-α than teetotalers, and this is generally associated with liver dysfunction (Fiers, 1991; Heberlein et al., 2014; Kapoor et al., 2021).

The macrophage-migration inhibitory factor (MIF) is a protein that controls both innate and acquired immunity that is increased in heavy drinkers. The dual inhibitor of phosphodiesterases 4 and 10 and of MIF, Ibudilast, exhibits good effects in patients with alcohol consumption problems and in animal models of alcohol dependence. Recent results suggest that MIF should be evaluated as a biomarker to predict the psychotherapeutic and pharmacological response in patients with alcohol consumption problems. It is considered a viable candidate for a therapeutic target (Petralia et al., 2020).

Prolonged consumption of ethanol alters the expression of neuroinflammation genes, either in the mRNA transcription process, at the post-transcriptional level or at the protein level. To achieve this, it can resort to the transfer of this type of molecule from the blood or those produced by astrocytes and brain microglia cells, the latter resident macrophages in the brain, the first line of defense. Hyperbranching of microglia can be caused by the increased release of proinflammatory cytokines and other signaling molecules in the body (He and Crews, 2008). There is a connection between some proteins in the brain and those in the blood, so their measurements could be used as a biomarker for the disease. The aim of this exploratory study was to determine if there was a difference in gene expression of some candidate genes associated with inflammatory response in blood when comparing samples from controls and cases.

MATERIAL AND METHODS

Definition of the subject of study.

Men and women over 18 years of age, born in Colombia, residents of the city of Bogotá, who were linked to the National University of Colombia, either as students, teachers, or administrative employees, and who agreed to participate by signing consent,

were included in the study. 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 validated for Colombia by Campo and Ospina (Campo-Arias et al., 2013; Ospina-Díaz et al., 2012). The selection of the sample of individuals was done for convenience. The control group was selected considering that they were people of the same sex and similar ages as those tested and that they obtained AUDIT scores less than 7, while the selection of individuals with problematic use of alcohol was conducted considering the following criteria: individuals whose first problem with addictive substances was alcohol and who obtained AUDIT scores greater than 7. On the other hand, all of them, both controls and cases, underwent a semi-structured interview, prepared based on Scoppeta and Silla (Silla Stoel and Rosón Hernández, 2009; Scoppetta, 2011) with the aim of obtaining more information about habits, frequency and amount of consumption, medical condition, personal history and family and thus be able to identify the phenotypes of interest and clearly define the control groups and cases. Volunteers who reported infections, allergies, some pharmacological treatment, diagnosed liver and psychiatric diseases were excluded.

From the study of single nucleotide variants, in which 139 people took part, 73 were classified as controls and 66 as people with problematic alcohol use (Rey M and Aristizabal FA, results not yet published). About 30 individuals with the lowest and highest AUDIT value were selected from each group for this study. The case-control type is a descriptive study with a non-probabilistic sampling for convenience, approved by the ethics committee of the Faculty of Medicine of the National University of Colombia. A critical aspect to better define the phenotype of interest was to calculate the grams of alcohol consumed by the study participants. Considering the type of alcoholic beverage, the volume consumed, and the degree of alcohol concentration in each type of beverage, the grams of alcohol taken on a regular day of alcohol consumption were calculated using the formula: grams of alcohol = (Volume(mL)X alcoholic strength of the drinkX0.8)/100 (Canadian Centre on Substance Use and Addiction, 2019).

Total RNA extraction

An aliquot of peripheral blood samples from the group of patients and controls, collected during the study period, was processed immediately after sampling to obtain mononuclear cells (PBMC), and the total RNA was extracted from the latter. Using Histopaque-1077 reagent following the manufacturer's instructions (Sigma-Aldrich), this density gradient procedure was carried out with minor adjustments (Sigma-Aldrich, n.d.). Finally, the mononuclear cell pellet was stored at -80 °C for total RNA extraction.

Total RNA extraction was performed using the RiboZolTM reagent (RNA Extraction Reagents) according to the manufacturer's instructions (AMRESCO-Sigma-Aldrich, Germany). The procedure was carried out in a special area of the laboratory, using decontaminated implements with the RNase Away reagent (Thermo Fisher Scientific, USA). The quantity and quality of the RNA was evaluated using spectrophotometric and electrophoretic methods, respectively. One microliter of total RNA was quantified in a spectrophotometer, paying special attention to the 260/280 ratio as a purity criterion that should be > or = 1.8. Agarose electrophoresis was performed with a loading buffer

containing 6M urea. The gels were stained with SYBR safe, where the 28S and 18S ribosomal RNA bands were visualized.

Reverse transcription reaction (RT)

Before starting RT, 8 µL of the total RNA obtained from each sample was treated with one µL of DNase I in 10X First Strand buffer (1 µL, in a total volume of 10 µL), for one hour at 37°C, to eliminate possible DNA contamination. The High-Capacity cDNA Reverse Transcription Kit was used to reverse transcription according to the manufacturer's instructions (Thermo Fischer Scientific, USA). Total RNA was added to the master mix in 10 µL with a concentration of 200 ng/µL. After mixing the ingredients, the tubes were put into a Bio-Rad T100 thermocycler programmed with the following conditions: a) 25°C for 10 minutes, b) 37°C for one hour, c) 85°C for five minutes, and d) 4°C thereafter.

Quantitative polymer chain reaction-qPCR.

For the qRT-PCR, primers were designed for the cDNA extracted from the messenger RNA (mRNA) of genes selected (Table 1). cDNA was amplified and counted in an RT-PCR reaction using a SYBR safe intercalating agent. The RT-PCR was performed in triplicate for each sample, and the Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as the reference gene and the positive control of the PCR (Foley et al., 1993).

Table 1. Characteristics of the primers used to perform qPCR and determine relative transcription of the selected genes.

Gene	Primers	Tm (melting temperature)	% GC	Size amplified in bp
	F:GCACTTGCTGGTGGATGTTC	59	55	
IL-6R	R:AGAGCACAGCCTTTGTCGTC	60	55	128
	F:AGAACCGCTCCTACAGCAAG	59	55	
MIF	R:GCGAAGGTGGAGTTGTTCCA	60	55	130
	F:GTTGTGCCTACCCCAGATTG	58	55	
TNFR1	R:CGATTTCCCACAAACAATGGAG	58	45	175
	F: GCCAAGGAGGGAGTTGTGGCTGC	68	65	
SNCA	R:CTGTTGCCACACCATGCACCACTCC	68	60	133
	F:CACCAGGGCTGCTTTTAACTCTGGTA	64	50	
GAPDH	R:CCTTGACGGTGCCATGGAATTTGC	65	54	131

The product was quantified by adding an intercalating agent (SYBR®safe) that binds to the amplicon quantitatively, so that the greater the product, the higher the fluorescence will be emitted. The analytical, quantitative method used was to measure the ΔΔCt. The Ct of the tested gene and the reference gene in each sample were directly compared, and then the ΔCt of the samples was measured. We chose the GADPH (glyceraldehyde 3 phosphate dehydrogenase) gene as the most appropriate reference for this type of experimental design, and the interplate calibrator sample was a mixture of equal proportions of controls and cases cDNA with a concentration of genetic material of 1 ng/uL(Bustin et al., 2009; Martínez-Rodríguez and Rey-Buitrago, 2020; Vargas Hernández and Rey Buitrago, 2020). For calculating the relative transcription (Roche, 2016), the

following equation was used: Expression Rate= ET(CtTCal-CtT) /ER(CtRCal-CtR) which corresponded to the calculation of relative transcription measures using a reference gene, where ET was the efficiency of amplification of the target gene; ER was the reference gene amplification efficiency; CtT was the quantification cycle of the target gene; CtR was the reference gene quantification cycle; Cal: interplate calibrator sample.

Before assembly of this assay, a pre-experimentation phase was conducted to evaluate several essential parameters for the validation of the results and with the purpose of standardizing the qPCR assay. The following preliminary tests were performed to determine the optimal primer concentration, primer binding specificity conditions, amplification efficiency, and dynamic range for each qPCR. Serial dilutions of the cDNA were used to evaluate its efficiency and dynamic range. Results indicated that the assay retained linearity in the concentration range between 0.25 and 12.5 ng/uL. Therefore, the protocol worked. The efficiencies of the assays for each gene were as follows: IL6R1: 2.08; TNFR1: 2.08; MIF: 1.83; SNCA: 2.18 and GADPH: 1.98. The values were within the accepted range of international qPCR execution protocols. To determine the optimum concentration of the primers, three values were used: 0.5, 0.25, and 0.125 uM. The test results indicated that the concentration of 0.125 uM generated melting curves with a single peak and efficiencies in the appropriate range (1.8-2.2). The specificity was obtained through the selection of the hybridization temperature of the primers.

The reaction was done with the Luna ® Universal qPCR Master Mix qPCR kit in a LightCycler® 96 thermal cycler. The composition of the qPCR assay was established and validated by our research group. The qPCR master mix (10X) was 1 uL, primer F and R (10 uM) were 0.125 uL each, and cDNA was between 1 and 3 uL (concentration of 1 ng/uL). To set up the qPCR reaction in the thermocycler, the following conditions were used: 1) one initial denaturation cycle (95°C, 300 seconds) 2) 45 cycles: Denaturation (95°C, 15 seconds), annealing and extension (65°C, 30 seconds) 3) one melting cycle (95°C, 10 seconds, 65°C for 60 seconds and 97°C for one second) and a final cooling cycle at 37°C. Each sample was run in triplicate and the average Ct of each of the genes measured and the normalizing gene in the control, cues and calibrator (interplate) samples was obtained. The minimum and maximum values, quartiles 1, 2 (median) and 3, and outliers were determined. A typical data set was found at 1.5 interquartile ranges, showing a maximum of three outliers in some of the genes studied, which were not used in the analyses. The Ct values for the GADPH normalizing gene did not present significant differences between the plates and the sample group. To verify the specificity of the assay, the melting temperature curves were run for five genes (SNCA, TNFR1, IL6R1, MIF, GADPH) where a single peak corresponding to the expected segment is evident, subsequently corroborated by electrophoresis. One band was observed in agarose gel.

ELISA assay

Four protein products, alpha synuclein (SNCA) were analyzed using the BioVision Incorporated human ELISA kit; tumor necrosis factor receptor 1 using the TNFRSF1A kit from AVIVA SYSTEMS; MIF protein using the MIF ELISA kit (HUMAN) from AVIVA SYSTEMS and interleukin 6 receptor 1 using the IL6R1 ELISA kit (HUMAN) from AVIVA SYSTEMS. The total RNA was obtained from the plasma for all tests, both in

controls and in cases, using a ficoll gradient for the isolation of mononuclear cells. The plasma thus obtained was stored at –80 °C until the moment of the ELISA assay.

According to the instructions of the manufacturers of each kit, the ELISA assays were developed. Concentration standards were prepared in 300 uL volume serial dilutions with concentrations ranging from 0 to 500 pg/mL for TNFRSF1A, 0 to 1,000 pg/mL for SNCA; 0 to 10,000 pg/mL for MIF and 0 to 4,000 pg/mL for IL6R1. These patterns were repeated in duplicate. Similarly, a pre-assay was carried out in 1:100 dilutions, 1:10, 1:2 of some samples of the controls and cases, randomly selected to establish the appropriate dilutions to obtain absorbances within the range obtained for the standards. All tests were done in three different ways. ELISA conditions were run according to the instructions of the suppliers.

Using a previous calibration curve with the data obtained from the standards, we were able to determine the protein concentration. A battery of standards was developed to cover the expected range of concentrations in the unknown samples. The regression curve was fitted using least squares regression methods. The concentrations of mRNA in monocytes and of selected proteins in plasma were presented as the mean +/- SD (standard deviation) of the determined concentration and, for the comparison between cases and controls, the parametric t test was used for equality of means or the non-parametric Mann-Whitney U test statistic. The Shapiro Wilk test was used to find out how the data was spread out when the analysis included less than 50 people (controls, n=25 and cases, n=25). In cases where the distribution was not normal, non-parametric tests were used. The Levene test was also used to assess the homoscedasticity of the variances (p=0.05). If the results were normal and homoscedastic, the two-factor ANOVA test was performed, and the Bonferroni post hoc test was used, when necessary. The box plots were constructed with the purpose of visualizing the differences between the groups of samples under analysis. A significance level of 0.05 was determined for all cases. The calculations were done with the program SPSS version 23.

RESULTS

Sociodemographic characteristics

Valid data were obtained from 50 individuals who participated in the expression study, 25 of whom were classified as controls and 25 as individuals with problematic alcohol use. Of the controls, 14 were women and 11 men. In the problematic alcohol-use group, seven were women and 18 men. In both groups, the age range was similar, with subjects aged between 18 and 37 years, and an average of 24 and 23.5 years for controls and cases, respectively. Approximately 90% of the participants in both the control group and the group with problem drinking began to drink alcohol before they were 18 years old. Both groups were mainly composed of university students and professionals working at the university, the latter belonging in a greater proportion to the control group. In the group of problematic alcohol consumption, undergraduate university students predominated more than 50% (Table 2).

Table 2. Sociodemographic characteristics of the study participants.

Parameter	Problematic Alcohol Consumption n (%)	Controls n (%)	Problematic Alcohol Consumption vs Controls (p Value)
Age: mean ±SD (years)	23.5±3.9	24.1 ±3.9	0.937#
Range(years)	18-35	20-37	
Sex Men	18 (72)	11 (44)	0.085
Sex Women	7 (28)	14 (56)	
AUDIT Score			
Range 0-7	0 (0)	25 (100)	0.000*
Range > 7	25 (100)	0 (0)	
Alcohol Consumption (g, grams)	209±92	37±25	0.000*
Age of onset of consumption de alcohol (years)			
<18	22(88)	24(96)	0.2971
>18	3(12)	1(4)	
Family members with consumption problems			
Yes	9(36)	12(48)	
No	0(0)	0(0)	0.390
Do not know, not answer	16(64)	13(52)	
Consumption of other psychoactive substances			
Yes	25(100)	12(48)	0.000*
No	0(0)	13(52)	
Socioeconomic level			
Low	7(28)	6(24)	0.948
Half	18(72)	18(72)	
High	0(0)	1(4)	

n= number of individuals
SD= standard deviation

Considering that the AUDIT questionnaire is a screening tool, we applied a semi-structured questionnaire to the participants while the blood sample was taken to better characterize and select our sample. The average amount of alcohol consumed during the last session was asked. Consumption was the closest to the sampling date (minimum abstinence of 48 hours), which reflected the participants' average consumption per event. About 50% of the controls mainly drank beer, while those with problematic alcohol consumption preferred to drink three different types of alcoholic beverages: beer, rum, and brandy. In the case group, the subjects drank an average of 209 g of alcohol per event, and in the control group, they drank an average of 37 g. 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. Most of the latter (>50%) could be classified as alcohol-dependent. Regarding the socioeconomic level, we did not find any differences between the two groups, presenting the same proportion of individuals of average condition as most of the individuals. Compared to the control group, 100% of individuals with problematic alcohol consumption were found to consume other types of psychoactive substances, such as marijuana and tobacco. Although the selection criteria stated that the first substance consumed was alcohol, consumers of other substances were not excluded due to the impossibility of finding exclusive consumers of alcohol.

Most of the cases were consumers of alcohol and other types of psychoactive substances. Finally, with the aim of having an approach to the hereditary factor, the question was asked about relatives with alcohol consumption problems. Furthermore, more than half of both cases and controls stated that they were unaware of a family member having alcohol consumption problems.

mRNA expression study

When all the participants were analyzed, significant p-values were observed in the expression of the TNFR1 and MIF genes. Those with problematic alcohol use showed a reduction of two and 1.5 times, respectively, in each gene, as shown in Table 3 and Figure 1. When it comes to the SNCA gene, there was a trend towards increased expression in individuals with problematic alcohol use, although the differences were not statistically significant. There is no difference in the expression of the IL6R1 gene between controls and cases. When comparing the samples by sex, similar results were observed: in the general sample, the relative expression of the MIF and TNFR1 genes decreased significantly in both sexes, while the relative transcription of the SNCA gene increased in men significantly (p=0.036) from 0.7 to 1.6 times, while there were no statistically significant changes in women (Figure 2). In the relative expression of the mRNA of the IL6R1 gene, no significant difference was identified between the groups analyzed.

Table 3. Comparison of means of the relative transcription of mRNA of the SNCA, IL6R1, TNFR1 and MIF genes in peripheral blood mononuclear cells of controls and alcohol-dependent cases and other parameters such as distribution and variance (normalizing gene-GADPH).

Gene	Statistical distribution (Shapiro-Wilk test) P-value, n	Variance comparison (Levene's test) P-value	Comparison of means (± SD) (T test or Mann-Whitney U test)	P-Value
SNCA n=50	Control: Not Normal 0.043*; n=25 Problematic Alcohol Consumption: Not Normal 0.013*; n=25	0.027*	1.02±0.74 1.42±1.17	0.193
IL6R1 n=48	Control: Normal 0.465; n=25 Problematic Alcohol Consumption: Normal 0.319; n=23	0.178	1.64±1.10 1.39±0.92	0.403
TNFR1 n=48	Control: Not Normal 0.043*; n=25 Problematic Alcohol Consumption: Normal 0.260; n=23	0.013*	0.99±0.065 0.53±0.36	0.007*
MIF n=50	Control: Not Normal 0.005*; n=25 Problematic Alcohol Consumption: Normal 0.319; n=25	0.023*	1.39±0.63 0.92±0.43	0.004*

n=sample size
*Significant p-values

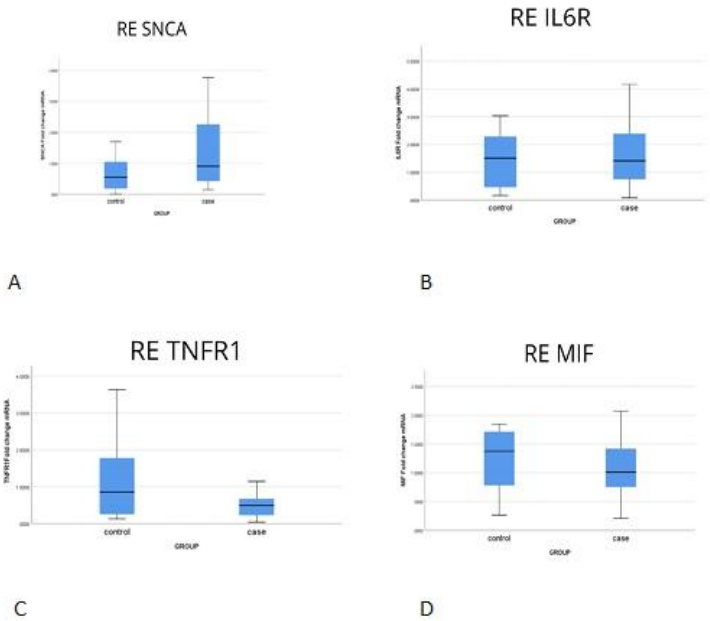

Figure 1. Relative expression mRNA (RE) of SNCA (A), IL6R1(B), TNFR1(C) and MIF in peripheral blood mononuclear cells in controls and cases with problematic alcohol use (normalizing gene: GADPH). The data is presented as a boxplot showing the range of values from 25 to 75%, with a cross line representing the median.

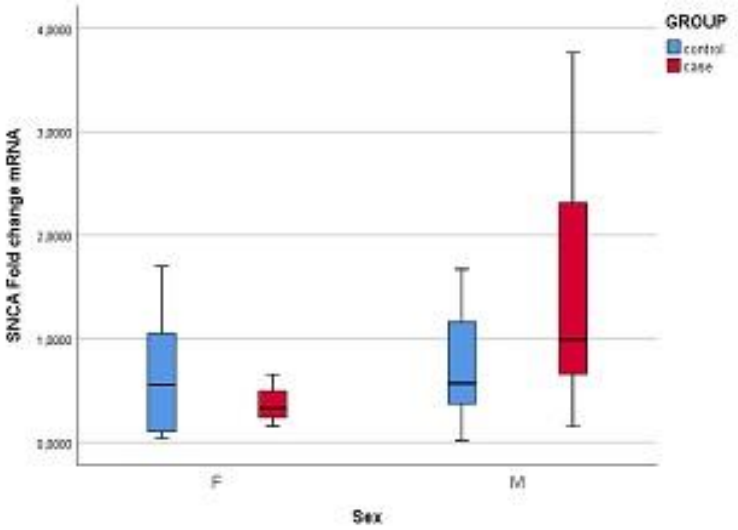

Figure 2. Realative expression of SNCA mRNA in peripheral blood mononuclear cells in controls and cases with problematic alcohol use (normalizing gene: GADPH) discriminated by sex (F: female, M: male). The data is presented as a boxplot showing the range of values from 25 to 75%, with a cross line representing the median.

Figure 3 illustrates an upward change in SNCA expression in individuals with problematic alcohol consumption, and a pronounced downward expression change in TNFR1 and MIF. The results obtained through normalized relative quantification with a

reference gene such as GADPH with the $2^{-\Delta\Delta Ct}$ method provide valuable information on the isolated behavior of each gene, and it is important to remember that these values are relative to the relative increase or decrease on the calibrator gene and not to the absolute expression of the genes tested.

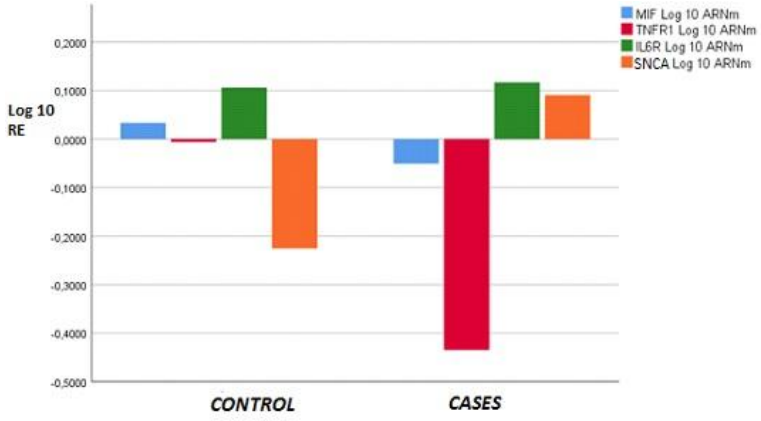

Figure 3. The base 10 logarithms of the relative mRNA transcription measurement of the selected genes: MIF (blue), TNFR1 (red), IL6R1 (green) and SNCA (orange). The normalizing gene is GAPDH.

When comparing the relative expression of the genes under study between the groups: cases and controls, the correlation analysis was performed. In the controls, a linear, positive and strong relationship can be seen between the expression of the pairs of genes with a high correlation coefficient (r): TNFR1-IL6R1 (r=0.819), MIF-IL6R1(r=0.716), TNFR1-SNCA (r=0.563), SNCA-IL6R1(r=0.586), which are reduced in all cases by more than 50% when correlating with the group of cases of problematic alcohol consumption. This suggests that there was a change in the relative transcription of one of the genes in the group of cases that changed the strong linear relationship between the two variables when compared to the controls. The correlation coefficient is high in both cases for the mRNA expression of a pair of genes: TNFR1-MIF with r=0.694 and r=0.756, respectively (see table 4 and figure A2).

Table 4. Matrix of correlation between the relative expression rates of the mRNA of the genes of interest (SNCA, IL6R1, TNFR1 and MIF) in the samples of the control groups and individuals with problematic consumption (cases).

Genes Of Interest	Group	SNCA Correlation coefficient	P-Value	IL6R1 Correlation coefficient	P-Value	TNFR1 Correlation coefficient	P-Value	MIF Correlation coefficient	P-Value
SNCA	Control	1	---	0.586	0.005*	0.563	0.008*	0.251	0.260
	Cases	1	---	0.560	0.013*	0.228	0.308	0.364	0.088
IL6R1	Control	0.586	0.005*	1	---	**0.819**	0.000*	**0.716**	0.000*
	Cases	0.560	0.013*	1	---	0.110	0.653	0.346	0.125
TNFR1	Control	0.563	0.008*	**0.819**	0.000*	1	---	**0.694**	0.000*
	Cases	0.228	0.308	0.110	0.653	1	---	**0.756**	0.000*
MIF	Control	0.251	0.260	**0.716**	0.000*	**0.694**	0.000*	1	---
	Cases	0.364	0.088	0.346	0.125	**0.756**	0.000*	1	---

*Significant p-values

Protein expression study

When comparing gene expression in the form of mRNA and protein, they tended to move in the same direction. Sometimes, after transcription and translation, the relationship between the gene and the protein can change. The protein factors SNCA, IL6R1 and TNFR1 showed no differences, while the MIF showed a twofold decrease in individuals with problematic alcohol use. This was achieved by analyzing the samples without any type of stratification (Table 5 and Figure 4). When the samples were stratified by sex, in SNCA, IL6R1, and MIF, the differences were evident in men, with a statistically significant decrease greater than 50% in SNCA, IL6R1, and MIF, respectively, and in women, only MIF showed a decrease of approximately 60% as in the general simple (Figure 5).

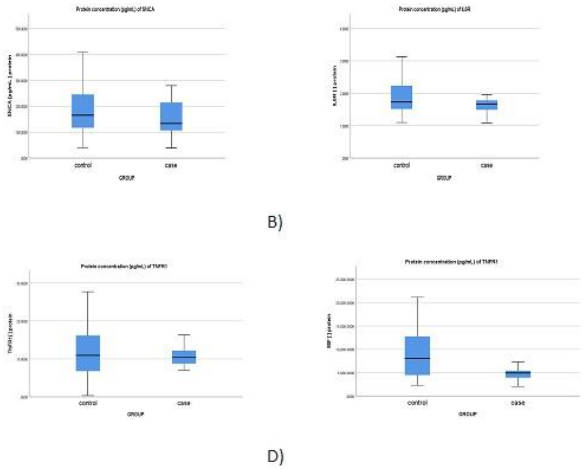

Figure 4. Protein concentration (pg/mL) of SNCA (a), IL6R1 (b), TNFR1 (c) and MIF (d) in blood plasma of controls and cases (individuals with problematic alcohol use). Data is presented as a boxplot showing the range of values from 25 to 75% with a cross line representing the median.

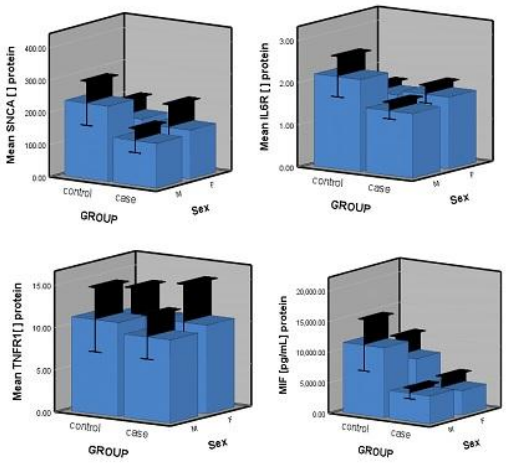

Figure 5. protein concentration (pg/mL) in plasma of controls and cases stratified by sex (M: male and F: female). With the letter, a. SNCA; b. IL6R1; c. TNFR1 and d. MIF.

Table 5. Comparison of mean protein concentrations of SNCA, IL6R1, TNFR1 and MIF in blood plasma of controls and cases with problematic alcohol use.

Protein	Statistical distribution (Shapiro-Wilk test) P-value; n	Variance comparison (Levene's test) P-value	Comparison of means or medians (pg/mL) (T test or Mann-Whitney U test)	P-Value
SNCA	Control: Normal 0.362; n=25	0.554	198.13±135.62	0.585
	Cases: Normal 0.170; n=25		199.31±165.77	
IL6R1	Control: Normal 0.123; n=25	0.010*	1.94±0.60	0.883
	Cases: Normal 0.559; n=25		2.15±1.08	
TNFR1	Control: Normal 0.435; n=25	0.035*	11.77±6.33	0.490
	Cases: Normal 0.066; n=25		9.90±4.34	
MIF	Control: Normal 0.071; n=25	0.000*	8898.34±5342.38	0.001*
	Cases: Normal 0.724; n=25		4391.42±1659.75	

n=sample size
*Significant p-values

When performing a correlation analysis between the concentration variables of the protein factors (SNCA, IL6R1, TNFR1 and MIF), none showed high coefficients. On the contrary, these indices were close to zero, which indicates the absence of high coefficients (Table 6).

Table 6. Matrix of correlation between the concentration of protein in plasma and the expression of the genes of interest in the samples of the control groups and alcohol abuse cases.

Genes Of Interest	Group	SCNA		IL6R1		TNFR1		MIF	
		Correlation coefficient	P-Value	Correlation coefficient	P-Value	Correlation coefficient	P-Value	Correlation coefficient	P-Value
SCNA	Control	1	---	-0.275	0.156	-0.048	0.801	0.269	0.150
	Cases	1	---	0.221	0.429	-0.082	0.746	0.007	0.976
IL6R1	Control	-0.275	0.156	1	---	0.065	0.743	0.008	0.968
	Cases	0.221	0.429	1	---	-0.095	0.746	-0.123	0.933
TNFR1	Control	-0.048	0.801	0.065	0.743	1	---	-0.237	0.200
	Cases	-0.082	0.746	-0.095	0.746	1	---	-0.189	0.426
MIF	Control	0.269	0.150	0.008	0.968	-0.237	0.200	1	---
	Cases	0.007	0.976	-0.123	0.933	-0.189	0.426	1	---

*Significant p-values

DISCUSSION

This is the first study in Colombia to compare the expression of four inflammatory response genes in a sample of individuals with problematic alcohol consumption to a control group. The control group had a balanced number of men and women, while the alcohol users had a larger number of men. This was expected because one of the main signs of alcohol dependence in women is that it is hidden. (Gómez Moya, 2006). Nearly 90% of the participants, both controls and cases, started drinking alcohol before the age of 18 (even

though the legal minimum age for alcohol consumption is 18 years), which confirms the trend observed worldwide for greater alcohol consumption in adolescents and children. This behavior is erroneously trivialized and naturalized. Likewise, in the problematic alcohol consumption group, students from undergraduate academic programs predominate, confirming the previously stated trend (Giménez-García et al., 2018; Marta Nayara Sanjuán Urrea, 2020). A significant difference was observed in the consumption of other psychoactive substances by the group of cases in relation to the controls (Table 2). Since it is practically impossible to find exclusive consumers of alcohol, the participants of the case group were classified as polyconsumers, since the cases with the first and predominant substance of consumption - alcohol - were selected. Recent data indicate that the most prevalent behavior in the world is the simultaneous use of various psychoactive drugs (polydrug use). In this regard, it should be noted that alcohol is present in 90% of polydrugs in different populations (Pascual et al., 2013).

It has been known for decades that alcohol consumption, whether acute or chronic, causes changes in gene expression, in the form of mRNA or proteins throughout the body and particularly in specific tissues, mainly the liver and nerves (Preedy et al., 1999). Continuous alcohol consumption increases the excretion of nitrogen, with the concomitant loss of mass, which can be of skeletal or cardiac muscle, among others. Also, qualitative changes in the products of protein expression in the liver, muscle and/or bone are observed. Acute in-vitro exposure to ethanol decreases the production of proinflammatory cytokines IL1, IL6 and TNF-α in monocytes and macrophages. On the other hand, prolonged exposure to alcohol in vivo has been linked to the activation of cytotoxic T cells (LTC) and natural killers (NK) and the increase in cytokines these types of cells make. Similarly, chronic drinkers have higher levels of proinflammatory cytokines, such as IL-6, IL-7, TNF-α, GM-CSF, and IFN-ϒ than controls. Heavy alcohol consumption increases the presence of infections and a poor response to them, with alterations in the levels of circulating cytokines. These are excellent candidates for biomarkers of alcohol dependence, although no consensus has been reached on their presentation patterns (Achur et al., 2010).

Determining the expression of proteins in a specific tissue shows us the real amount that has been synthesized at that specific moment in the life of that tissue under study. Most laboratory tests confirm the correlation between mRNA expression data and their corresponding proteins, the latter being more informative (Fei, 2011). In this study, we found that the transcription rates of the SNCA and IL6R1 genes did not vary when comparing individuals with problematic use of alcohol with controls, although in SNCA a tendency to increase transcription was observed in individuals with problematic use of alcohol without Significant p-values, although if there were differences when comparing men, an increase in cases was shown. Various authors have shown both an increase and a decrease in SNCA concentrations in individuals with problematic use of alcohol and alcohol dependence. These results could be explained by analyzing the study design, depending on the correct assignment of the phase in the addiction process in which the patient is, the tissue being measured, the current age and the start of alcohol consumption, the sex, etc. (Bönsch et al., 2004; Guillot et al., 2015). In relation to this last parameter, we found an altered concentration of the protein products of SCNA, IL6R1 and MIF in men with problematic alcohol consumption, with a decrease of more than 50%. Also, the transcription rates of the TNFR1 and MIF genes were found to be altered, with a decrease in individuals with problematic alcohol use. When measuring the concentration of the protein product, the

same trend was observed only in the MIF protein in the general sample without stratifying. When the sample was analyzed overall, no changes were observed in the TNFR1 protein, and no changes were observed when the sample was stratified by sex. It is important to note that there were changes in the expression of SNCA in men, but the changes were opposite. In these cases, there was a rise in the quantity of mRNA of SNCA, whereas the concentration of protein-SNCA was lower.

The analysis of mRNA and protein expression in mononuclear cells showed that other cells that make these cytokines are changing their plasmatic concentration. Another possible, less likely explanation would be the difference in expression in the form of mRNA and protein of the same gene, which could be clarified by post-transcriptional, translational, and post-translational regulation phenomena. Several mechanisms may be involved, including the alteration of the expression of the SNCA protein in neurodegenerative diseases in humans and animal models by microRNAs such as miRNA-7, miRNA-153 and miRNA-433 (Lugli et al., 2008; Tarale et al., 2018).

Differential expression of MIF has been observed in brain regions, epithelium, endothelium and peripheral blood lymphocytes. MIF is an upstream cytokine regulator involved in many inflammatory and immune diseases. MIF is known to directly activate the expression of TNF-α, IL2, IL8 and INFϒ (Tahara, 2009) and MIF inhibition substantially reduces the synthesis of these four interleukins (Bucala, 2013). There have been reports that MIF has increased among alcohol consumers, especially heavy drinkers, contrary to what was found in this study. However, it is important to note that most of the participants in this study were young university students who were not considered to be heavy drinkers. As shown in Table 4 and Figure A2, we found a strong correlation between the expression of MIF mRNA and TNFR1 mRNA. Likewise, the expression of their protein products is correlated. There is a clear relationship between gene variants, repeat -794 CATT5-8 (rs5844572) and SNV -173 G>C (rs755622) and transcription rates, the long forms being more transcriptionally active (7 and 8 repetitions) than the short ones (≤5) and the C variant than G, polymorphisms that were not evaluated in this work, although it would be worth having this information in the future (Janeczek et al., 2014, 2015).

Chronic ethanol consumption causes an increase in TNF-α, whereas acute consumption causes a decrease. This increase may be due to elevated levels of the AP1 transcription factor that binds to the promoter region of the TNF-α gene, as well as missing the repression of the SMAD 3 and 4 proteins that also interact with this promoter region of the gene (Zhao et al., 2008). In this work, we found a 50% decrease in the expression of the TNF-α receptor type 1 (TNFR1) in monocytes in people with problematic alcohol use. Considering that the form that we determined of this receptor is soluble and that its interaction with TNFα- exerts an action that apparently decreases the levels of TNF-α in the blood (sequesters it). There would be 50% fewer soluble receptors in consumers and by decreasing their presence, it would appear to contribute to an increase in the concentration of TNF-α, a variable that we did not measure in our study, which is reported to increase.

On the other hand, the receptor could be involved in a desensitization process, decreasing its presence and therefore its synthesis. This is a mechanism for protecting cells from the prolonged action of the TNF-α ligand due to its prolonged action (Idriss and Naismith, 2000). A difference was found in the relative transcription of the TNFR1 gene, which decreased by almost 50% in alcohol-dependents, but this was not reflected in the concentration of proteins that remained the same in alcohol-dependents. Perhaps we can

identify a post-transcriptional regulation event that could include a decrease in the stability of TNFR1 mRNA in individuals with problematic alcohol use or epigenetic regulation events such as the presence of miRNA-19 and miRNA-103a type microRNAs, which have been found altered in various addictive pathologies and related to the regulation of the expression of genes such as TNF-α, TNFR1, TNFR2, CFLIP, TRADD, CASP3 and CASP8 (Nunez and Mayfield, 2012). It would be intriguing to further investigate the microRNA profiles in patients experiencing problematic alcohol use.

TNFR1 and MIF were the most interested genes, which showed differences between controls and cases. These biomarkers play a role in processes such as regulation of macrophage differentiation, apoptotic signal activation, protein denidylation, cellular response to mechanical stimuli, and macrophage differentiation among others (Sprowl et al., 2012; Wang et al., 2013). Their effects can be very different because they are synthesized by various cell types. In this work, we found that these two markers were less expressed than they were in other studies. Such discrepancies with other studies may be related to the methodological approach and the type and size of samples used to assess monocyte functional status (i.e., animal models vs. human studies, in vitro vs. ex vivo, use of different tissue targets, the use or not of different stimulus conditions, the status of ethanol intake at the time of the study, active alcohol dependence versus the period of abstinence), the nutritional status of the patients, and the presence or absence of alcoholic liver disease or some type of liver damage not yet known or diagnosed at the time of the interviews. One could also speculate that these findings are more closely aligned with acute consumption in those instances, as opposed to chronic and high consumption.

(Laso et al., 2007; Petralia et al., 2020). Since most of the participants were young students, the results also reflect a more adaptive response of people who have just started to consume alcohol.

However, we found significant differences in the expression of the genes at the protein level depending on the sex of the study participants: in men with problematic alcohol consumption, three genes (SNCA, IL6R1 and MIF) decreased in their relative expression, respectively, while in women only MIF. It is clear that there are sex differences in gene expression, just as there are other types of biochemical or physiological differences between men and women. In the last few years, there has been more evidence that different pathologies have differences that cannot be explained only by hormonal differences. Sex is a risk factor in the development of alcohol dependence, as it is the most important genetic factor in the case of men, while environmental factors in the case of women. In our study, these genes are related to inflammatory and immune responses that present clearly differentiated expression patterns between the sexes (Natri et al., 2019; Prescott, 2002; Weijers et al., 2003).

To summarize, similar to other investigations, we observed disparities in the expression of certain inflammatory response genes when comparing controls and cases. We found that the TNFR1 and MIF genes decrease in mRNA in people who have problems with alcohol consumption, but the MIF protein only decreases. However, the expression of the SNCA, IL6R1, and MIF proteins varied depending on the sex of the participants, with men showing lower levels of expression than women. In addition to the sex of the participants, these variables also depend on the stage of the disease, with clear differences between those who are in the initial phase and heavy drinkers. These findings suggest that

SNCA, IL6R1, and MIF are candidates for further study, increasing the sample size, and suggesting that they could be therapeutic targets in alcohol-related diseases.

Supplementary data to this article can be found online at

https://repositorio.unal.edu.co/handle/unal/84041

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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