Plasma concentrations of Granulocyte Colony-Stimulating Factor (G-CSF) in Patients with Substance Use Disorders and Comorbid Major Depressive Disorders

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Research Article

Keywords: G-CSF, major depressive disorders, substance use disorders

Posted Date: February 12th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-152636/v1

Abstract

Aims: *Granulocyte colony–stimulating factor* (G-CSF) has raised much interest due to its role to cocaine addiction in preclinical models. We analyzed the circulating expression of G-CSF in abstinent chronic users of alcohol and/or cocaine with or without comorbid major depressive disorders to investigate the role of this trophic factor with complicated substance use disorders.

Methods: We recruited 176 patients and 136 controls. Patients were divided in 50 patients with major depressive disorder (MDD) and 126 abstinent substance use disorders (SUD) patients undergoing treatments for alcohol (N=66) or cocaine (N=60) addiction according to DSM-IV-TR criteria. A blood sample was collected to examine plasma concentrations of G-CSF.

Results: The plasma concentrations of G-CSF were significantly decreased in the cocaine group compared with the SUD control group. There was a sex dimorphism in the alcohol group, with lower G-CSF concentrations in women compared with men. Plasma concentrations of G-CSF were associated with abstinence and with the length of alcohol problems. The decrease in G-CSF was associated with comorbid MDD, a finding specific for SUD patients since there were no alterations of G-CSF primary settings MDD outpatients.

Conclusions: Circulating G-CSF is reduced in SUD patients, being associated to comorbid MDD. A sex-dependent effect was observed in female AUD. Plasma G-CSF concentrations might be used as a predictor of length of chronic alcohol use and as a stratification role in the dual diagnosis in SUD. Further investigation is needed to explore the role of G-CSF as potential biomarker of pathogenic/prognosis in SUD population.

Introduction

Substance use disorders (SUD) are chronic neurobiological-based medical illness contributing to almost 6% of all deaths worldwide [1]. In the last years, it has been confirmed that SUD is associated with inflammation in the central nervous system (CNS), a process that can cause long-term behavioral alterations by activating glial cells, mainly microglia and astrocytes, and modifying the plasticity of the central nervous system [2,3]. Best known neuroinflammation-inducing drugs are alcohol and cocaine, and multiple studies are addressing now how these inflammatory changes contribute to the clinical course of SUD and associated pathologies [3–6].

Considering alcohol, it has been described that its pharmacological actions in the CNS include multiple neurotoxic effects as activation of microglial cells, alterations in the release of several mediators such as *brain derived neurotrophic factor* (BDNF) [4], modifications of the lipid membrane causing alteration of the cell membrane permeability, promoting changes in proliferation and maturation of neurons, and eventually neuronal apoptosis [5–7]. Interestingly, alcohol directly induced the activation of pro-inflammatory immune signaling, as revealed by the characterization of *toll-like receptor 4* (TLR4)

dependency of the glial activation responses associated with alcohol exposure [8]. This finding supported the inflammatory view of alcohol-induced toxic effects: preclinical studies based in reward and in alcohol withdrawal behavioral responses have revealed that there were several pro-inflammatory mediators involved in the regulation of alcohol effects [8–11]. As an example, the chemokine *monocyte chemoattractant protein-1* (MCP-1) secreted by glial cells was found associated with alcohol consumption and alcohol-related neurodegeneration [12,13]. Other studies monitoring plasma concentrations of immune mediators showed alcohol-induced deregulation of certain chemokines related with immune responses such as SDF-1 [*stromal derived factor* (CXCL12)] and *fractalkine* (CX3CL1) that were found to be decreased in patients with alcohol use disorders (AUD) [14]. Finally, important neurotrophic factors such as BDNF and *insulin-like growth factor-1* (IGF-1) were found to be profoundly affected in abstinent AUD patients [4,15].

Regarding cocaine, a stimulant substance with an elevated potential of abuse contributing to a major health problem and social costs [16], it is known that its consumption is associated with neuronal cytotoxicity to neurons and altered neurotrophic responses, through a nitric oxide-mediated signaling pathway [17]. However, despite cocaine use disorders (CUD) are mostly characterized by dysfunctions in reward-related brain circuits, deregulating mesolimbic dopaminergic reward pathways and glutamate receptor-dependent signaling cascades [18,19], multiple studies also describe immune signaling alterations associated to its consumption [8]. Regarding circulating immunological mediators related with cocaine consumption, cytokine *tumor necrosis factor alpha* (TNF-α) was found to be elevated in plasma while the anti-inflammatory *interleukin 10* (IL-10) was found to be decreased and linked to cocaine-related chronic stress [20]. Moreover, circulating concentrations of *Interleukin 1β* (IL1β), *fractalkine* and SDF-1 were found to predict severity of cocaine use disorder, suggesting a relevant contribution of the immune system to cocaine addiction [21].

The contribution of the immune system is not only associated to the severity of addiction or its toxic effects, but also it might foster the development of additional co-morbid disorders. Substance use disorder patients are more likely to suffer comorbid psychiatric disorders through their lives than the general population [22,23], affecting the course of the psychiatric illness [24]. Mood, anxiety, psychosis and personality disorders are the most prevalent diagnosis found in SUD population [25–27]. In this sense, immune signaling deregulations have been found to be associated with some psychiatric disorders such as major depressive disorder (MDD) [28,29], something also described in SUD patients [13].

The search for immune mediators participating in the natural history of addiction has brought to place new actors such as *Granulocyte colony–stimulating factor* (G-CSF),a glycoprotein known by its hematopoietic functions [30]. As a neurotrophin, its main functions include the proliferation and differentiation of myeloid progenitors [30], promoting the generation of granulocyte precursors and anti-apoptotic actions [31,32]. G-CSF receptors were studied in the CNS due to their neuroprotective properties [33] including its anti-inflammatory effects found in dopaminergic neurons in the study of neurodegenerative

disorders such as Parkinson's [34] and Alzheimer disease [35]. Regarding addiction, G-CSF was found to be a relevant signal that promotes cocaine self-administration and consolidates cocaine seeking behavior [36]. However the presence of alterations of this trophic immunomodulatory factor in SUD has not been studied in humans.

The impact of addictive disorders in the health system, highlight the importance of effectives prevention programs and treatments [37]. Because several preclinical and clinical studies have reported an inflammatory state in SUD population, and taking on account the above described role in preclinical models of cocaine addiction, we explored the hypothesis that the circulating concentrations of the growth factor G-CSF are altered in abstinent chronic SUD patients (alcohol or cocaine users) with or without comorbid MDD. The confirmation of this hypothesis might help to further understand the contribution of immune-derived mediators to substance use disorders.

Materials And Methods

Participants and recruitment

This exploratory study is composed by 312 volunteers, including a sample of 176 patients divided in 2 cohorts, one of 50 patients with major depressive disorder (MDD) recruited from primary-care settings at Spain National Public Health System, and 125 abstinent SUD patients undergoing outpatient treatments for alcohol (N = 65) and cocaine (N = 60) problems. After the psychopathological evaluation the abstinent patients were further divided following the diagnosis of specific substance use disorders (SUD).

A sample of 136 healthy subjects was recruited from two different sources: a multidisciplinary staff cohort of volunteers working at the *Hospital Regional Universitario de Málaga* (Málaga, Spain) and a second cohort obtained from volunteers donating data and plasma to the Banco Nacional de ADN Carlos III (Salamanca, Spain). Control sample was divided in two groups [SUD control (N = 92) and MDD control (N = 44)] matching the clinical sample by age, sex and BMI ratio. Patients were recruited at the addiction treatments from *Hospital Regional Universitario de Málaga* (Málaga, Spain), *Centro Provincial de Drogodependencias* (Málaga, Spain) and *Hospital Universitario 12 de Octubre* (Madrid, Spain) while control participants were included from databases of healthy subjects from *Hospital Regional Universitario de Málaga* (Málaga, Spain).

The participation in the study was voluntary and had to meet eligibility based on inclusion criteria: both genders ≥ 18 years old up to 65, and the following diagnosed based on DSM-IV-TR criteria: major depressive disorder diagnosis with at least two months of depressive symptoms for the MDD group, substance use disorder diagnosis with AUD and/or CUD for the SUD group. The exclusion criteria included: personal history of long-term inflammatory diseases or cancer, severe mental disorders precluded evaluation, pregnant or breast-feeding women, at least 4 weeks of abstinence in the SUD group, any substance use disorders (except nicotine and caffeine in the MDD group and infectious diseases.

With regard to the control groups, the inclusion criteria were being matched with the SUD or the MDD group by age and BMI ratio and having no diagnosis or medication for psychiatric disorders.

Ethics statements

Written informed consents were obtained from each participant after a complete description of the study. All the participants had the opportunity to discuss any questions or issues. The study and protocols for recruitment were approved by the *Ethics Committee of the Hospital Regional Universitario de Malaga* in accordance with the Ethical Principles for Medical Research Involving Human Subjects adopted in the Declaration of Helsinki by the World Medical Association (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and Recommendation No. R (97) 5 of the Committee of Ministers to Member States on the Protection of Medical Data (1997), and Spanish data protection act [Regulation (EU) 2016/679 of the European Parliament and of the Council 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC (General Data Protection Regulation). All collected data were given code numbers in order to maintain privacy and confidentiality.

Clinical assessments

All the participants in the study were evaluated using structured interviews by trained and experienced psychologists. In the SUD cohort, psychopathological disorders were diagnosed according to the DSM-IV-TR criteria [38] using the Spanish version of the *Psychiatric Research Interview for Substance and Mental Disorders* (PRISM) [38,39]. The PRISM is a semi-structured interview with good psychometric properties in the evaluation of substance use disorders and in the main psychiatric comorbid disorders related to substance use population [39,40]. In the major depression cohort the Beck Depression Inventory-II (BDI-II) was used to assess the severity of depression [41]. Control subjects were evaluated using the Spanish version of the *Composite International Diagnostic Interview* (CIDI) for the detection of psychiatric disorders [42].

Collection of plasma samples

Blood extractions were conducted in the same conditions by experienced nurses in the morning after fasting for 8–12 hours. Venous blood samples were extracted into 10-ml K2 EDTA tubes (BD, Franklin Lakes, NJ, USA) and to obtain plasma, samples were centrifuged at 2200 g for 15 minutes (4°C). Plasma was individually assayed by three rapid tests for detecting infectious diseases: HIV (Retroscreen HIV, QualPro Diagnostics-Tulip Group Ltd, Goa, India), hepatitis B (HBsAg Test, Toyo Diagnostics-Turklab Inc., Izmir, Turkey) and hepatitis C (Flaviscreen HCV, QualPro Diagnostics-Tulip Group Ltd). Infected samples were discarded following the laboratory safety protocols. Each plasma sample was registered and characterized and were stored at 80°C until determination.

Determinations of G-CSF

Plasma concentrations of G-CSF were determined using a selective enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions: human colony stimulating factor 3 granulocite

(CSF3) ELISA Kit (#CSB-E04563h 96T, Cusabio, Houston, Tx, USA). To perform the ELISA protocols we used 100µL of the samples into each cell, the plates were incubated 90 minutes at 37ºC. Subsequently, it was washed twice with the washing buffer, 100µL of a solution of anti-G-CSF antibodies linked with a biotin molecule were added and it was subjected to a new incubation period of 60 minutes at 37ºC. The plate was washed three times, a further 100µL of a Streptavidin-HRP containing solution in 1:1000 dilution was added, and incubated again at 37°C for 30 minutes. After this period of time, it was washed five times, 90µL of the TMB substrate solution in a 1:1000 dilution was added and it was incubated at 37ºC for other 15 minutes, which was the time necessary to observe blue color in the samples and in the standard curve. At this time the reaction was stopped with a H2SO4 solution and the absorbance was measured at 450nm. In all cases, internal controls and calibration curve and were included in each ELISA Kit.

Statistical analysis

Data in the tables are expressed as number and percentage of subjects [N (%)] or mean and standard deviation (SD). The significance of differences in categorical and normal continuous variables was determined using Fisher's exact test (chi-square test) and Student's *t*-test, respectively.

Analyses of covariance (ANCOVA) and analyses of variance (ANOVA) were performed to indicate the impact of the independent factors (i.e. main substances in treatment, lifetime SUD diagnosis, lifetime MDD…) controlling for additional variables as sex and age to control on the G-CSF plasma concentrations as dependent variable. The *post hoc* tests for multiple comparisons were performed using Tukey's correction test. The estimated marginal means [95% confidence intervals (95%CI)] were described and represented in the figures. Correlation analyses were performed using the Pearson's coefficient (r). The statistical analyses were carried out with the GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA), and IBM SPSS Statistical version 22 (IBM, Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

Results

Socio-demographic description of the sample

This study included 312 participants divided into SUD and MDD populations and a health group of controls matched by age and BMI with their reference group. **Table 1** shows the socio-demographic variables of the sample participants. The mean age of the cocaine group was 35.4 years, and the 30% were women, with secondary education degree (76.7%). Significant differences have been found in age between substances (p<0.001); being the patients of alcohol group those who seek medical help later than the cocaine group. Moreover, were found differences between the SUD group and MDD group in the sex-balance proportion, with more women in the MDD group.

TABLE 1. Socio-demographic variables of the study groups

		SUD N=125		MDD N=50	Healthy group N=136
		Alcohol N=65	Cocaine N=60		
Age (Mean ± SD)		49.4 ± 6.6	35.4 ± 7.8	43.8 ± 8.8	41.7 ± 11.1
BMI Kg/m^2 (Mean ± SD)		26.1 ± 4.7	24.3 ± 3.8	24.8 ± 4.1	24.8 ± 3.9
Sex [N (%)]	Women	23 (35.4)	18 (30.0)	40 (80.0)	79 (58.1)
	Men	42 (64.6)	42 (70.0)	10 (20.0)	57 (41.9)
Education [N (%)]	Elementary	27 (41.5)	8 (13.3)	7 (14.0)	14 (11.1)
	Secondary	23 (35.4)	46 (76.7)	25 (50)	51 (40.5)
	University	15 (23.1)	6 (10.0)	18 (36)	61 (48.4)
Employment [N (%)]		26 (40.0)	27 (45.0)	31 (62.1)	105 (77.2)

Abbreviations: BMI=body mass index; MDD=major depressive disorder; SUD=substance use disorder.

Plasma G-CSF concentrations in relation to history of substance use disorders

The impact of the history of substance use disorders in the plasma concentrations of G-CSF was firstly investigated using a two-way ANCOVA with the group [healthy group (N=92) and SUD (N=125)] and sex as factors and age as covariate. The multiple comparisons revealed that plasma concentration of the G-CSF was significantly affected by the history of SUD [$F_{(1,212)}$ = 5.915; p=0.016]. There was a significant reduction of G-CSF plasma concentrations in the SUD group [1414.267 (95%=1095.285-1733.248) pg/mL] compared with the healthy group [1999.588 (95%CI=1650.209-2348.967) pg/mL]. There was no effect found when the factor sex was included in the analysis, nor SUD x sex interaction in the plasma concentration of G-CSF, indicating a major effect of drug consumption on the circulating levels of G-CSF. These results were represented in **Figure 1A**.

Because the effect found in the previous comparison, we investigate the impact of the main substances in treatment. We use a two-way ANCOVA with the group [healthy group (N=92); cocaine group (N=60) and the alcohol group (N=65)] and sex as factors and age as covariate. Plasma concentration of G-CSF was affected by the group [$F_{(2,210)}$ = 3.168; p=0.044] with a significant reduction of G-CSF plasma concentration in the cocaine group [1256.372 (95%CI=768.468-1744.277) pg/mL] compared with the healthy group [1985.995 (95%CI=1641.795-2330.195)] (see **Figure 1B**). Interestingly, there was found an interaction effect between SUD and sex [$F_{(2,210)}$ = 3.393; p=0.035]. The *post hoc* test revealed a significant difference (p=0.002) between the G-CSF plasma concentrations in men from the alcohol group [2221.328 (95%CI=1683.462-2759.193) pg/mL] compared with women from the alcohol group [1015.682 (95%CI=304.437-1726.926) pg/mL] and significant differences (p=0.006) found between healthy women and women from the alcohol group [1994.955 (95%CI=2488.334-1501.575) pg/mL and 1015.682 (95%CI=304.437-1726.926), respectively]. These results were represented in **Figure 1C**.

Interestingly, when we compare with the patients with both substance use disorders (alcohol and cocaine), the effects seen above lost their significance, suggesting that the combination of both drugs abolished the differences observed in primary CUD/AUD patients. We use a two-way ANCOVA with the group [healthy group (N=92); CUD group (N=51); AUD group (N=50) and PoliUD group (N=24)] and sex as

factors and age as covariate. There were no effects found by the group neither the interaction between group and sex. In **Supplementary S1** were represented the estimated marginal means of the plasma G-CSF concentrations based in the history of substance use disorders.

Plasma G-CSF concentrations in relation to substance-use characteristics

Since there were found differences on G-CSF plasma concentration according to the history of substance use disorder (either alcohol or cocaine), we explore additional relevant variables related to the specific disorder group, such as severity, length of abstinence or years of SUD diagnosis. To this end SUD patients were divided according to alcohol or cocaine group for correlation analysis (**Table 2**). At the time of the evaluation, the alcohol group had a mean of 7.8 DSM-IV-TR alcohol disorder criteria and an average length of 160 days of abstinence with a positive correlation of G-CSF plasma concentration with the length of alcohol abstinence. The cocaine group had a mean of 7.6 DSM-IV-TR cocaine disorder criteria and an average of 55.2 days of abstinence at the moment of the evaluation. We did not found any correlation between CUD-related variables and the G-CSF plasma concentrations in the CUD group.

TABLE 2. Correlation between G-CSF concentrations and substance-use variables

Variables	Alcohol N=65		Cocaine N=60	
	rho	*p*-value	rho	*p*-value
DSM-IV-TR criteria [1-11]	-0.092	0.466	-0.239	0.066
Length of abstinence	0.283	**0.022**	0.032	0.813
Years of SUD diagnosis	0.127	0.314	-0.076	0.518

Bold values are statistically significant for p<0.05 after Spearman's correlation.

Plasma G-CSF concentrations in relation to psychiatric disorders

Since SUD is often associated with psychiatric co-morbidity, we analyzed whether G-CSF concentrations might be different regarding the presence of psychopathology. Our SUD sample was characterized to display an elevated prevalence of psychiatric disorders. The 51.6% of the SUD sample has other mental comorbid diagnosis, being the 31.7% mood disorders, 27% anxiety disorders, 15% cluster B personality disorders and 4.8% psychotic disorders, the most prevalent. No statistical differences found in the sex proportion between them. In **Table 3**, was described the clinical comorbid characteristics according to alcohol and cocaine group.

TABLE 3. Clinical characteristics of the SUD group

Psychopathological evaluation [N (%)]	Alcohol N=65 [N (%)]	Cocaine N=60 [N (%)]	*p*-value
Major depressive disorder (MDD)	29 (44.6)	11 (18.3)	**0.002**
Dysthymia disorder	2 (3.1)	3 (5.0)	0.672
Cyclothymic disorder	1 (1.5)	1 (1.7)	-
Schizophreniform disorder	-	1 (1.7)	0.480
Psychotic disorder not specified	5 (7.7)	1 (1.7)	0.210
Social phobia	2 (3.1)	3 (5.0)	0.670
Panic disorder	10 (15.4)	2 (3.3)	**0.032**
Agoraphobia disorder	1 (1.5)	2 (3.3)	0.670
Generalized anxiety disorders	3 (4.6)	2 (3.3)	1.000
Obsessive-compulsive disorder	1 (1.5)	6 (10.0)	0.054
Post-traumatic stress disorder (PTSD)	7 (10.8)	6 (10.0)	1.000
Anorexia disorder	1 (1.5)	10 (16.7)	**0.003**
Bulimia disorder	3 (4.6)	16 (26.7)	**0.001**
Antisocial personality disorder	3 (4.6)	2 (3.3)	1.000
Borderline personality disorder	10 (15.4)	5 (8.3)	0.277
Attention deficit hyperactivity disorder	6 (10.2)	10 (16.7)	0.421

As shown in the psychopathological description of the SUD sample, we examined the effect of the most prevalent disorders in our abstinent SUD sample in the G-CSF plasma concentrations using two-way ANCOVAs.

The two-way ANCOVA showed a main effect in the diagnosis of MDD in the abstinent SUD patients, but there were no other significant effects found in the G-CF plasma concentrations in the other comorbid diagnosis according to the psychopathological evaluation (see **Supplementary S2**).

Because the 66.7% of the abstinent SUD group was using psychoactive medication during the last 12 months: antidepressants (40.7%), anxiolytics (40.7%), *disulfiram* (39%), anticraving (22.8%), and antipsychotics (8.9%), respectively, we monitored the effect of this medication on G-CSF levels. The

analysis revealed no effect of medication in the G-CSF plasma concentrations. The differences in G-CSF concentrations based on the psychiatric medication of the SUD sample are described in **Table 4**.

TABLE 4. Plasma G-CSF concentrations in the SUD group according to psychiatric medication

Variables	SUD N=125		Statistics (1)		
	Non medicated	Medicated	F	df	*p*-value
	Mean 95%CI	Mean 95%CI			
Antidepressants	1566.51 [1173.88-1959.14]	1265.11 [790.70-1739.53]	0.939	1,121	0.335
Anxiolytics	1495.95 [1102.07-1889.83]	1368.13 [892.21-1844.05]	0.168	1,121	0.683
Anticonvulsants	1426.41 [1080.97-1771.86]	1503.63 [867.33-2139.94]	0.045	1,121	0.833
Antipsychotics	1359.36 [1045.25-1673.47]	2305.69 [1303.40-3307.97]	3.182	1,121	0.077
Disulfiram	1418.50 [1029.71-1807.29]	1483.82 [997.83-1969.81]	0.043	1,121	0.836

(1) *Data were analyzed by ANOVA and *p<0.05 denote a significant main effect of group factor.*

Plasma G-CSF concentrations in relation to comorbid MDD in substance use disorders

As indicated in the previous section, the association between the presence of comorbid MDD in abstinent SUD patients and G-CSF plasma concentrations was investigated using a two-way ANCOVA with the group [SUD with MDD (N=40) and SUD without MDD (N=85)] and sex as factors and controlled for age. We found a clear effect of MDD diagnosis on circulating G-CSF concentrations [$F_{(1,120)}$ =6.568; p=0.012] with a significant decrease in SUD patients with MDD diagnosis [833.727 (95%CI=289.896-1377.558) pg/mL] compared with SUD group with no comorbid MDD [1722.798 (95%CI=1327.901-2117.695) pg/mL]. Interestingly, an interaction effect between MDD and sex [$F_{(1,120)}$ = 4.055; p=0.046] was observed. The *post hoc* corrected Tukey test revealed a significant difference (p=0.017) between the G-CSF plasma concentrations in no comorbid MDD women compared with women with comorbid MDD.

SUD patients diagnosed with MDD were divided according to DSM-IV-TR criteria into primary and substance-induced MDD. The impact of the type of comorbid MDD in SUD patients was studied using a one-way ANOVA but there were not found statistical differences in the plasma concentrations of the neurotrophin G-CSF (see **Table 5**) regarding the origin of the MDD diagnosis.

TABLE 5. Plasma G-CSF concentrations in the SUD group according to the type of comorbid depression

	SUD sample (N=125)				Statistics [1]		
	No MDD (N=85)	Primary MDD (N=15)	Induced MDD (N=16)	Both MDD (N=9)	F	df	*p*-value
	mean 95%CI	mean 95%CI	mean 95%CI	mean 95%CI			
G-CSF plasma concentrations	1715.51 [1356.89-2074.12]	808.19 [-45.49-1661.83]	1358.99 [532.45-2185.55]	517.69 [-584.38-1619.76]	2.396	3,121	0.072

[1] *Data were analyzed by ANOVA and *p<0.05 denote a significant main effect of group factor.*

Moreover, to investigate the impact of comorbid MDD in the different groups we separate the sample by the main substance in treatment. Regarding the alcohol group, a one-way ANOVA with the group [MDD (N=29); no MDD (N=36)] showed that plasma concentration of G-CSF was affected by the MDD diagnosis [$F_{(1,63)}$= 4.327; p=0.042] with a reduction of G-CSF plasma concentration found in alcohol patients with comorbid MDD [1008.093 (95%CI=344.885-1671.301) pg/mL] compared with alcohol with no comorbid MDD [1935.686 (95%CI=1340.438-2530.933) pg/mL]. These results were represented in **Figure 2**. In contrast, in the cocaine group there were not found differences in the plasma concentrations of G-CSF in the comorbid MDD diagnosis.

Finally, we analyzed if the plasma concentrations of G-CSF were affected by the current psychiatric medication in the alcohol group, but the analyses did not showed any statistical differences in the G-CSF levels in the alcohol patients receiving medication (see **Supplementary S3**).

Plasma G-CSF concentrations in relation to major depressive disorders

Because there was some evidence that peripheral alterations of this glycoprotein could be related with the presence of comorbid MDD in SUD population, we investigated the plasma concentrations of G-CSF in primary-care patients diagnosed with depression (MDD) with no substance use disorders. A two-way ANCOVA was performed with the group [Healthy group (N=44) and MDD group (N=50)] and sex as factors and controlled for age. We did not found any significant effects from group, age, sex, or either the interaction between factors (**Figure 3A**), suggesting that is the concurrence of SUD + MDD the origin of the specific decrease in circulating levels of this immunomodulatory trophic factor.

The MDD group was using psychiatric medication during their treatment, mostly antidepressants (36%) and anxiolytics (40%). Moreover, and based in recent research in our group that found n-acylethanolamide levels elevated in MDD patients with antidepressant treatment medication [43], we decided to investigate the possible effects of antidepressant and anxiolytic medication in the primary-care patients diagnosed with depression (MDD) with no substance use disorders. The analysis revealed no significant effect based on the current psychiatric medication treatment in G-CSF plasma concentrations. The marginal means were represented in **Figure 3B**.

Discussion

In the present study, we examined the *granulocyte colony–stimulating factor* (G-CSF) in the peripheral plasma concentrations of abstinent patients with alcohol or cocaine use disorders and in a cohort of major depressive disorder patients, who were recruited from active programs in treatment primary-care settings. Data were compared with healthy controls. Additionally, all these participants were characterized through psychopathological assessment based on the DSM-IV-TR criteria. The main findings of this study suggest that: 1) The plasma concentrations of G-CSF was significantly decreased in patients requesting treatment for cocaine use disorders, compared with the SUD control population of healthy people; 2) A history of AUD also affected G-CSF. In this case, there was a sexual dimorphism in the plasma concentrations of G-CSF in the alcohol group, with lower plasma levels in women compared with men; 3) The plasma concentrations of G-CSF were associated with the length of abstinence in the SUD groups; 4) The plasma concentrations of G-CSF were associated with the age, the length of abstinence and the years of diagnosis in the alcohol group; 5) G-CSF plasma concentrations were further decreased in the AUD patients with comorbid MDD disorders suggesting a possible potential role of this immunotrophic factor in dual diagnosis rather than in primary depression disorders.

The present results confirm that in addition to its known role as a modulator of myeloid cells from the bone marrow, G-CSF might contribute to the history of addiction in both AUD and CUD patients, although the nature of this contribution demands further research. While in animals this immunotrophic signal helps to consolidate cocaine reward/cocaine seeking behavior, in abstinent humans we observed that G-CSF concentrations are decreased in SUD, with an almost significant negative correlation with the severity of CUD. In the absence of data on acute cocaine effects on circulating G-CSF, we can only speculate on the nature of this finding. If the role of this factor in preclinical models of cocaine addiction is to boost/sustain cocaine seeking behavior [36], it is reasonable to think that after prolonged exposure to cocaine and abstinence, this factor might be down regulated in severe addicted patients, following the allostatic model set in place for most of the biological modulatory transmitters on addiction [44]. This finding demands to be conclusively determined. Regarding alcohol, we do not have information on the role of G-CSF in animal models of alcohol addiction, but our results clearly support that this immunotrophic factor might contribute to alcohol abuse and dependence, as it does with cocaine. Furthermore, this analyte has also been studied related to memory functions in rats, showing that the deficiency of G-CSF concentrations in hippocampus decreased spatial learning performance and memory formations [45]. Therefore, we consider that it would be interesting to measure the role of this growth factor in a neuropsychological cohort of patients with substance use disorders and substance-induced memory deficits. This is especially relevant in the case of alcohol, since this drug has been linked clearly to memory deficits and deceased circulating neurotrophic factors.

Another important aspect of the present study is the existence of sex-related differences in circulating G-CSF, a fact of interest for gender-specific stratification and interventions in addiction. The sex is an important physiological factor studied as an underlying susceptibility, with differential outcomes and treatment in biomedical research [46,47]. The literature describe sexual dimorphism in many alcohol-derived consequences, either psychiatric disturbances and injuries in the organ system [48]; suggesting that

women are more sensitive to alcohol behavioral outcomes whereas men may be more sensitive to the alcohol neurophysiologic effects [49]. Moreover, considering the variables related with the alcohol use disorder diagnosis, in our study we did not found differences in the length of AUD diagnosis neither less duration of alcohol use in women but in the number of abstinence periods achieved after the years. This result is in accordance with previous studies searching sex differences reporting that lifetime prolonged alcohol abstention is more common among women, although this difference is strongly influenced by socio-cultural factors [50].

Regarding the differences found in the growth factor G-CSF attributable to the interaction of the sex and the alcohol use disorders; we observe a sexual dimorphism response, with decreased G-CSF plasma concentration in female AUD patients. Accordingly to these sex differentially affected results, other preclinical studies have found sex-differences in the neuro-regulation, microglia signaling, reward processes and homeostasis in brain development [51,52]. There were found sexual dimorphism in pro-inflammatory mediators in chronic alcohol consumption; with a differential action in the immune system and a marked suppression of immune signaling in males in corticosterone response, concretely in *transforming growth factor β-1* (TGFβ-1) and *interleukin 6* (IL-6) [53]. Moreover, other cytokines as *interleunkin-1β* (IL-1β) and *tumor necrosis factor α* (TNF-α) were found up-regulated in females mice compared with males [54]. In the same way, studies in SUD population found differences between sexes in circulating factors, such *C-reactive* protein in the alcohol consumption [55]. Regarding the cocaine use, concentrations of G-CSF were affected by the lifetime CUD, although the sexual dimorphism has not been clearly found between them. Moreover, the absence of sexual differences could be supported by previous clinical studies indicating that chronic cocaine intake could attenuate sex-differences in the immune system expression [56].

On the other hand, epidemiological studies showed a bidirectional relation between SUD and comorbid disorders with genetic vulnerability as a common etiology factor [57,58] and sex differences observed in the prevalence of psychiatric comorbid disorders in SUD population [59]. Despite the elevated rates of comorbid mood and anxiety disorders found in our sample, we did not found differences between men and women. However, follow-up studies pointed that there were sex-differences in the course of comorbid affective disorders in AUD population, with more therapeutic need and a worse prognosis in short-term outcomes in male than female patients [60].

We demonstrated a positive correlation of plasma concentration of G-CSF with the length of alcohol abstinence and the years of diagnosis problems. As in the case of the growth factor G-CSF, other regulated markers were found related with the length of alcohol abstinence as the fatty acid derivative *oleoilethanolamide* (OEA). OEA is a satiety factor regulated downward as the length of abstinence increase in patients with alcohol use disorders acting as a marker of abstinence [61]; while G-CSF seems to behave as a possible predictor of chronic alcohol use.

Finally, our results showed that G-CSF plasma concentrations were decreased in abstinent AUD patients with comorbid MDD disorders. There are long-lasting stress effects studied in the development of mood disorders [62] with several deregulations produced on the pro-inflammatory status [63]. Recent studies in our group confirmed differences found in other peripheral mediators related to the presence of comorbid psychiatric disorders in AUD population; the circulating levels of the chemokine *eotaxin-1* (CCL11) were found decreased in AUD patients with mood and anxiety comorbid disorders [14]; and additionally, other peripheral plasma levels of acyl-glycerol concentrations [*2-araquidonil-glycerol* (2-AG) and *2-linoleoyl-glycerol* (2-OG)] were found decreased in AUD patients with comorbid anxiety disorders [26].

Conclusions and limitations of the present study

These findings support the importance of monitoring G-CSF in the context of substance use disorders and psychiatric comorbidity, but we are aware of the limitations of this study: 1) There are uncontrolled social variables from the patients SUD cohorts that can be considered as a source of variability. It is important to replicate these findings in other cohorts from different geographical and cultural backgrounds. 2) There are statistical limitations related to parametric assumptions, the sample size and the number of independent variables in the linear models that precluded the inclusion of more covariates (principally socio-demographic variables); 3) Larger samples of female substance use disorders patients and additional experimental groups should be included in further studies to reproduce these results and include more relevant covariates; 4) Longitudinal studies are also needed to monitor changes in this growth factor during acute exposure, active consumption and along abstinence if possible in the same SUD patients.

In conclusion, these findings support an effect of substance use disorders in G-CSF plasma circulating concentrations, with a significant decrease in the CUD sample and a sexual dimorphism in the plasma concentrations of G-CSF in abstinent AUD patients with lower levels in women. Interestingly, we found decreased plasma concentrations of G-CSF associated with comorbid lifetime major depressive disorders diagnosis in AUD patients. Conversely, these findings in comorbid MDD were not observed in abstinent CUD patients. Additionally, the plasma concentrations of G-CSF correlated with alcohol related variables as the length of abstinence, an interesting variable related with a good prognosis of the substance use disorders. Moreover, the last part of the study in patients with MDD demonstrated that the deregulation of G-CSF plasma concentration was related with the combination of AUD and comorbid MDD rather than in primary MDD. Further research is necessary to elucidate the role of G-CSF as a potential biological marker of neurotrophic state in dual diagnosis.

Declarations

Funding

The present study has been supported by the following programs and research projects: Subprograma Redes Temáticas RETICS (Red de Trastornos Adictivos RD12/0028/0021, RD16/0017/001 and,

RD17/0017/0003) funded by Instituto de Salud Carlos III (ISCIII), Ministerio de Economía y Competitividad (MINECO) and the European Regional Development Funds/European Social Fund (ERDF/ESF); Proyectos de Investigación en Salud (PI16/01698, PI17/02026, PI19/01577, PI20/0883) funded by ISCIII and ERDF/ESF; Proyectos de Investigación en Drogodependencias (PND2017/043, PND2018/033, PND2018/044 and PND2019/040) funded by Delegación del Gobierno para el Plan Nacional sobre Drogas, Ministerio de Sanidad, Secretaría de Estado de Sanidad and ERDF/ESF; Proyecto de Investigación en Salud (PI-0140-2018) funded by Consejería de Salud y Bienestar Social, Junta de Andalucía and ERDF/ESF. NGM holds a “Sara Borrell” research contract (CD19/00019) funded by ISCIII and ERDF/ESF. FJP and AS hold a “Miguel Servet II” research contract (CPII19/00022, CPII19/00031 respectively) funded by ISCIII and ERDF/ESF.

Acknowledgements

The authors are grateful to G. Rubio, J.J. Ruiz, C. Sánchez and M. Soria, for their valuable assistance throughout the clinical part of the study.

Conflict of interest declaration

None

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Figures

Figure 1

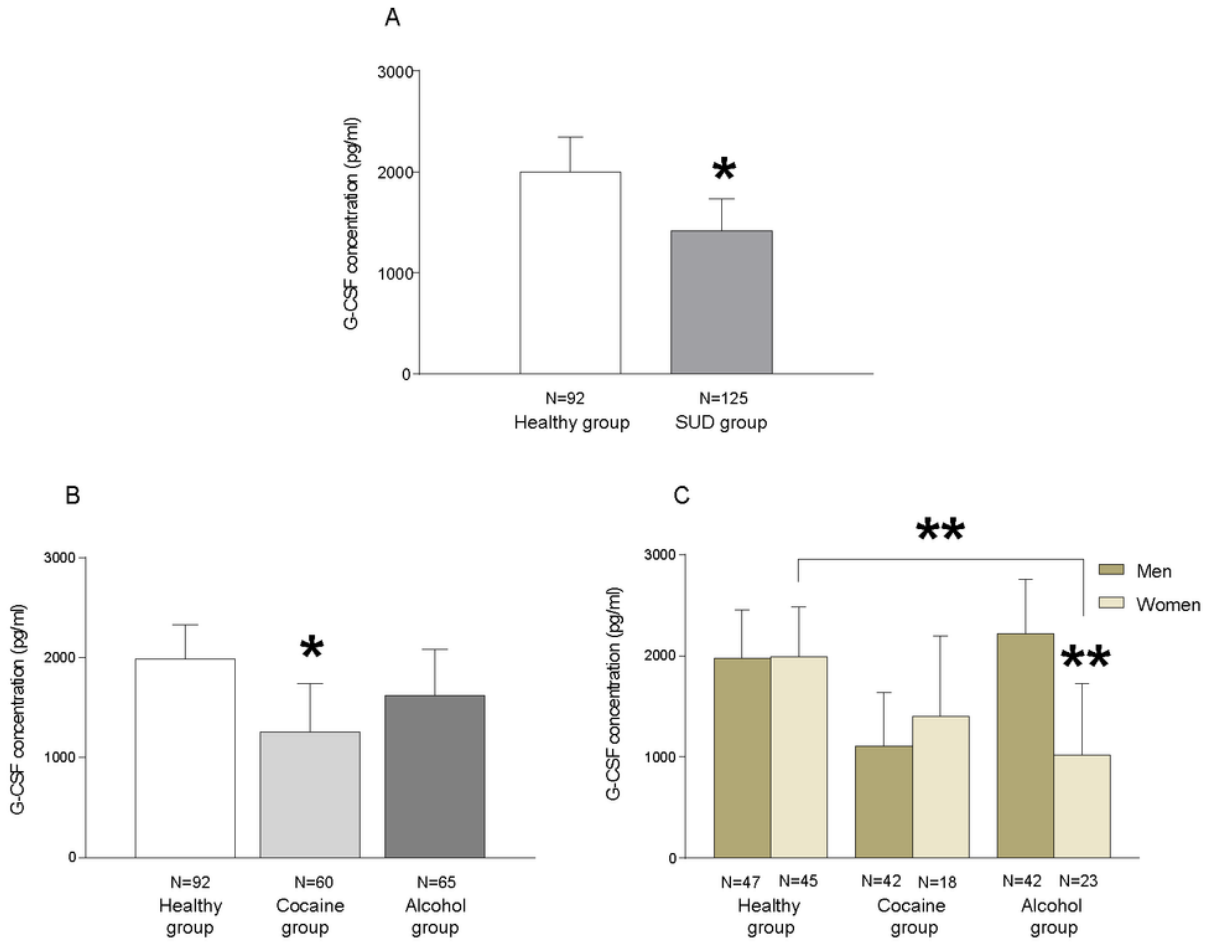

Figure 1

Plasma concentrations of G-CSF according to history of SUD. (A) Bars are estimated marginal means and confidence intervals (95%) representing G-CSF (pg/ml) according to the SUD group.; (B) Bars are estimated marginal means and confidence intervals (95%) representing G-CSF (pg/ml) according to the main substance in seeking treatment; (C) Bars are estimated marginal means and confidence intervals (95%) representing G-CSF (pg/ml) according to the main substance in seeking treatment and according to the SUD group and sex. Data were analyzed by a two-way ANCOVA and *p<0.05 **p<0.01 and ***p<0.001 and denote a significant main effect according to history of SUD and sex.

Figure 2

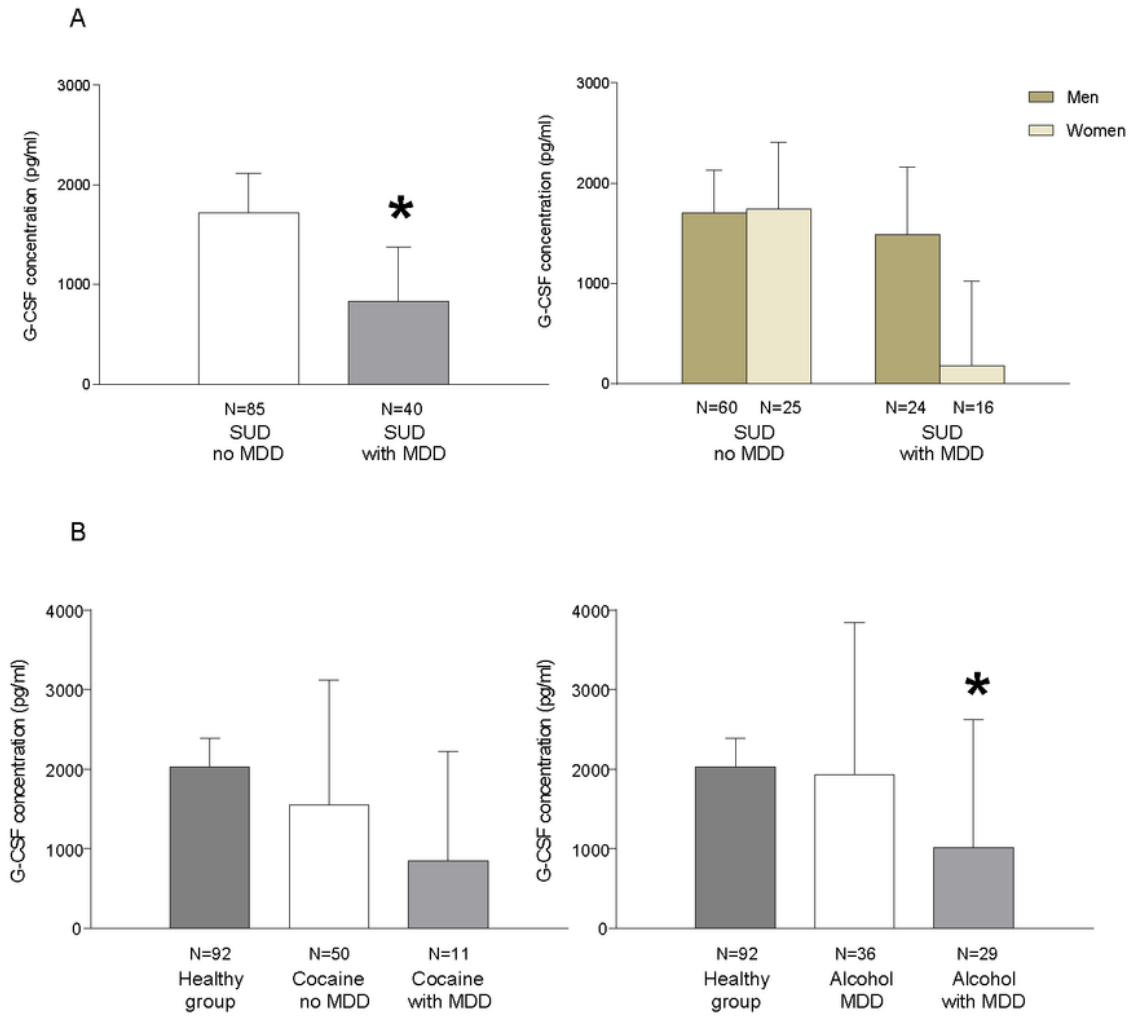

Figure 2

Plasma concentrations of G-CSF according to comorbid MDD in SUD. (A) Bars are estimated marginal means and confidence intervals (95%) representing G-CSF (pg/ml) according to MDD comorbid disorders in the SUD group and according to the MDD comorbid disorders in the SUD group and sex; (B) Bars are estimated marginal means and confidence intervals (95%) representing G-CSF (pg/ml) according to MDD

comorbid disorders in the cocaine group and in the alcohol group. Data were analyzed by a one-way ANOVA and *p<0.05 and denote a significant main effect according to comorbid MDD.

Figure 3

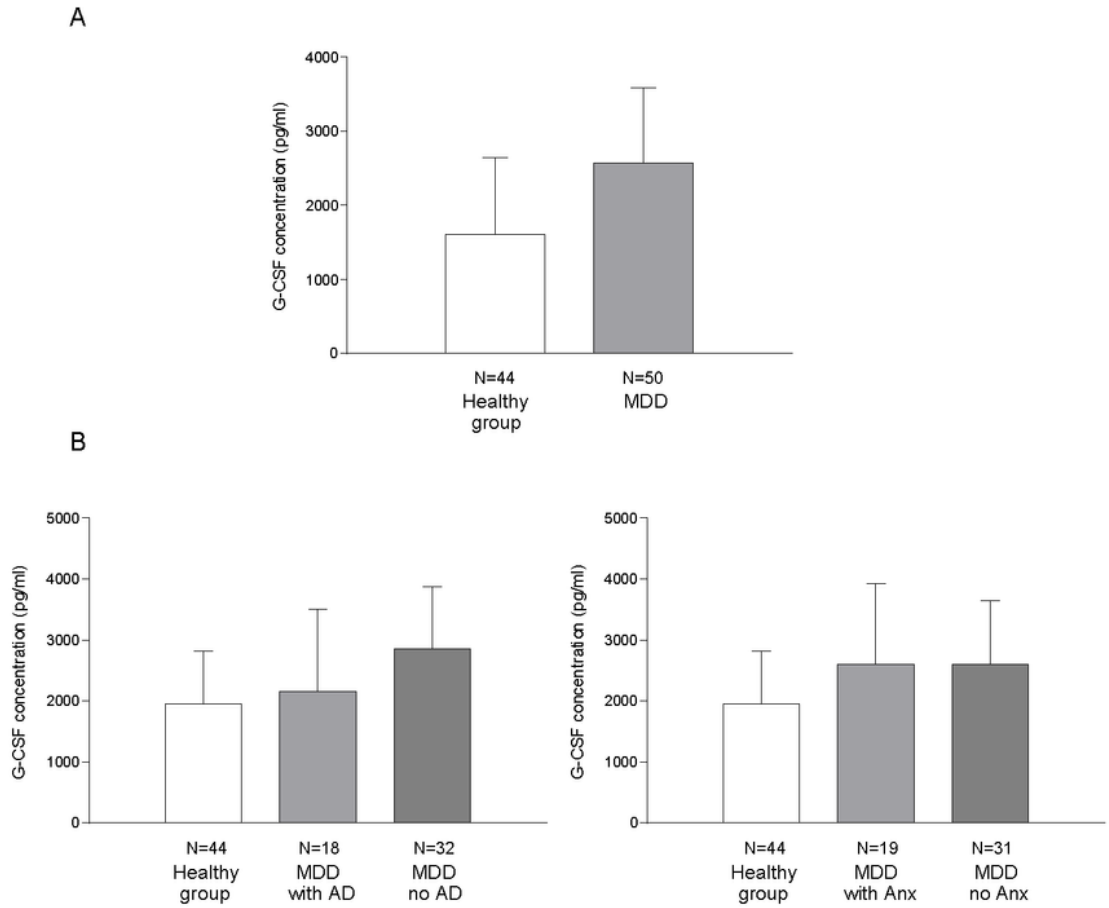

Figure 3

Plasma concentrations of G-CSF in alcohol study according to MDD. (A) Bars are estimated marginal means and confidence intervals (95%) representing G-CSF (pg/ml) according to MDD group; (B) Bars are estimated marginal means and confidence intervals (95%) representing G-CSF (pg/ml) according to

Antidepressant medication in MDD group and according to anxiolytic medication in the MDD group. Data were analyzed by a one-way ANOVA and *p<0.05 and denote a significant main effect.

Supplementary Files

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