Short Communication

Title: *CXCL12* rs1029153 polymorphism is associated with the sustained virological response in HIV/HCV-coinfected patients on HCV therapy

Running Title: *CXCL12* rs1029153 SNP and HCV therapy

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Character count of Title: 129
Character count of Running Head: 36
Word count of Abstract: 239
Word count of Keywords: 6
Word count of Text: 2535
Count of References: 24
Count of Tables: 2
Count of Figures: 1

ABSTRACT

The immune response against HIV and HCV infection partly depends on chemokine-mediated recruitment of specific T cells. CXCL12 polymorphisms have been associated with AIDS progression and survival, but there are not data related to HCV infection. The aim of this study was to evaluate whether *CXCL12* polymorphisms are related to achieve sustained virological response (SVR) after HCV therapy with pegylated-interferon-alpha/ribavirin (pegIFNα/ribavirin) in HIV/HCV-coinfected patients. We carried out a retrospective study in 319 naïve patients who started HCV treatment. The *CXCL12* (rs266093, rs1029153 and rs1801157) and *IL28B* (rs12980275) polymorphisms were genotyped by using GoldenGate® assay. Genetic data were analyzed under additive inheritance model. The overall rates of the SVR was 54.9% (175/319), 41.5% (90/217) in GT1/4 patients and 83.2% (84/101) in GT2/3 patients. Patients with favorable *CXCL12* rs1029153 T allele had higher SVR rate than patients with rs1029153 CC genotype (44% CC, 49% CT, and 61.3% TT; p= 0.025). No significant results for rs266093 and rs1801157 polymorphisms were found. Patients harboring the favorable rs1029153 T allele had significantly increased odds for achieving SVR (adjusted odds ratio (aOR)= 1.55 (95% of confidence interval (95%CI)= 1.01; 2.40); p= 0.047). Moreover, no significant association was found when the study population was stratified by HCV genotype (data not shown), possibly due to the low number of patients in each group. In conclusion, In this study, we found favourable *CXCL12* rs1029153 T allele seems be related to achieve SVR in HIV/HCV-coinfected patients on pegIFNα/ribavirin therapy.

Keywords: AIDS; chronic hepatitis C; Sustained virological response; SNPs; chemokines; HCV therapy

Hepatitis C virus (HCV) infection is considered a leading cause of chronic liver disease ranging from mild chronic hepatitis to end stage cirrhosis and hepatocellular carcinoma. In human immunodeficiency virus (HIV)/HCV coinfected patients, the combination antiretroviral therapy (cART) has made of chronic hepatitis C (CHC) an important comorbidity and a major cause of death [1].

The dual therapy with pegylated-interferon-alpha plus ribavirin (pegIFNα/ribavirin) is still used in HIV/HCV-coinfected patients for the treatment of CHC [2]. The sustained virological response (SVR) is achieved in approximately 20-40% of patients infected with HCV genotype 1/4 (GT1/4) and 50-60% in HCV genotype 2/3 (GT2/3) [2]. Today, the best baseline predictors for pegIFNα/ribavirin therapy include the age, sex, HCV genotype, HCV viral load, liver fibrosis, and the single nucleotide polymorphisms (SNPs) around *interleukin 28B* (*IL28B*) gene [3].

Nowadays, the use of new direct-acting antivirals (DAAs) has improved the SVR rate, particularly in difficult-to-treat patients infected with GT1/4 [2]. However, despite the emergence of DAAs, pegIFNα/ribavirin continues to be used alone or in combination with DAAs [2]. Besides, the new DAAs are more expensive and there are serious restrictions for its administration in many regions. For these reasons, treatment with pegIFNα/ribavirin remains the only option of therapy for many patients in the world.

The immune response against HCV infection depends partly on chemokine-mediated recruitment of specific T cells to the liver [4]. CXCL12, also known as stromal-derived factor 1 alpha (SDF1α), is the specific ligand for CXCR4 and CXCR7 [5].The CXCL12/CXCR4 axis has been besieged by many pathogens that employ a range of strategies to modify or exploit CXCR4 activity. While CXCL12/CXCR4 has been identified as a critical co-factor for entry of HIV into CD4+ T cells early on, other viruses may utilize CXCR4 to gain cell entry as well, modulate CXCR4 expression or alter its functional activity, with direct effects on cell trafficking, immune responses, cell proliferation, and cell survival [5]. In HCV infection, the CXCL12/CXCR4 axis has an important role in recruitment and retention of immune cells in the liver [6], and it has been associated with developing fibrosis, cirrhosis and hepatocellular carcinoma during CHC [6-9]. Moreover, the CXCR7-CXCL12 complex may act in two ways, as a modulator of CXCR4 signaling when acts as either a scavenger of extracellular CXCL12, and promoting the migration and chemotaxis when there is heterodimerization of CXCR7 with CXCR4 [5].

Few studies have linked *CXCL12* polymorphisms with viral infections. The *CXCL12* rs1801157, the best-studied polymorphism in *CXCL12* gene, has been associated with effects ranging from strong protection [10, 11] to enhancement [12, 13] of AIDS progression. Besides, rs1801157 polymorphism did not seem to have an effect on the susceptibility to HIV-1 infection [14]. There are not data about *CXCL12* SNPs and CHC.

The aim of this study was to analyze whether *CXCL12* polymorphisms are related to achieve SVR after HCV therapy with pegIFNα/ribavirin in HIV/HCV-coinfected patients.

We carried out a retrospective study in all HIV/HCV-coinfected patients who started treatment with pegIFNα/ribavirin on regular follow-up from October 2000 to June 2010

at two reference HIV hospitals located in Madrid (Spain): Hospital Gregorio Marañón and Hospital Carlos III. All patients were European whites.

The criteria for starting HCV antiviral treatment were: A) Inclusion criteria: CHC, no clinical evidence of hepatic decompensation, detectable HCV RNA by polymerase chain reaction, negative for hepatitis B surface antigen, CD4+ lymphocyte count higher than 200 cells/mm^3, and stable cART for at least 6 months before study entry or no need for cART according to treatment guidelines used in the study period. Furthermore, we included only HIV-infected patients who had an available DNA sample for DNA genotyping. B) Exclusion criteria: Active opportunistic infections, active drug or alcohol addiction, and other concomitant diseases or conditions such as diabetes, nephropathies, autoimmune diseases, haemochromatosis, primary biliary cirrhosis, Wilson's disease, a1-antitrypsin deficiency and neoplasia. Finally, we included only 319 patients who had an available DNA sample and genotyping data.

The study was conducted in accordance with the Declaration of Helsinki and patients gave their written consent for the study. The Institutional Review Board and the Research Ethic Committee of the *Instituto de Salud Carlos III* (ISCIII) approved the study.

HCV infection was documented in all patients by enzyme-linked immunosorbent assay (ELISA) and PCR test. HCV genotype was determined by hybridization of biotin-labeled PCR products to oligonucleotide probes bound to nitrocellulose membrane strips (INNO-LiPA HCV II, Innogenetics, Ghent, Belgium). Plasma HCV-RNA viral load was measured by polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor Test, Branchburg, NJ, USA) and real-time PCR (COBAS AmpliPrep/COBAS TaqMan HCV test); and results were reported in terms of international units per milliliter (IU/mL), with a lower limit of detection of 10 IU/mL.

Liver fibrosis was assessed by different methods, depending on the Hospital: i) Hospital Gregorio Marañon employed liver biopsy; and fibrosis score was estimated following the criteria established by the METAVIR Cooperative Study Group: F0, no fibrosis; F1, portal fibrosis; F2, periportal fibrosis or rare portal-portal septa; F3, fibrous septa with architectural distortion but with no obvious cirrhosis (bridging fibrosis); and F4, definite cirrhosis. ii) Hospital Carlos III used transient elastometry (FibroScanR, Echosens, Paris, France); and liver stiffness values ≤7.0, between 7.1 and 9.4, between 9.5 and 12.4, and ≥12.5 were considered to correspond with Metavir scores F0-F1, F2, F3, and F4, respectively.

Treatment regimens included pegIFNα 2a or 2b at standard doses (180 μg/week or 1.5 μg/kg/week, respectively) plus weight-adjusted ribavirin dosing (1000 mg/day for patients weighing <75 kg and 1200 mg/day for patients weighing ≥75 kg). Following international guidelines [15], patients with HCV genotypes 1 or 4 received either 48 or 72 weeks of treatment, and patients with HCV genotype 2 or 3 were treated for 24 or 48 weeks, depending on the virological response at week 4. A SVR was defined as an undetectable serum HCV-RNA level (<10 IU/mL) at week 24 after the end of the treatment.

We have analyzed the most common SNPs included into CXCL12 family, using the databases of HapMap Project (http://snp.cshl.org/cgi-perl/gbrowse/hapmap_B35/) and NCBI (dbSNP) (http://www.ncbi.nlm.nih.gov/entrez/). The selection criteria

were: i) SNPs located at 3′ untranslated region (UTR); ii) allelic frequency greater than 20% in European people. In summary, four SNPs (rs3740085, rs266093, rs1029153 and rs1801157) were genotyped.

Genomic DNA was extracted from peripheral blood with Qiagen kit (QIAamp DNA Blood Midi/Maxi; Qiagen, Hilden, Germany). DNA samples were sent at the Spanish National Genotyping Center (CeGen; http://www.cegen.org/) for genotyping of *CXCL12* (rs3740085, rs266093, rs1029153 and rs1801157) and *IL28B* rs12980275 polymorphisms by using GoldenGate assay with VeraCode Technology (Illumina Inc., San Diego, California, USA). The rs3740085polymorphism failed to have valid genotypes and was discarded for further analysis.

Statistical tests were performed with the Statistical Package for the Social Sciences (SPSS) 19.0 software (IBM Corp., Chicago, USA). All p-values were two-tailed and statistical significance was defined as $p<0.05$. Data were analyzed by on-treatment analysis of observed data. Patients that prematurely interrupted their HCV treatment due to adverse events, abandonment, or loss of follow-up were discarded from the analysis.

The genetic analysis was carried out according to an additive inheritance, which was the model that best fitted our data according to the statistical power to detect significant associations. The chi-squared/Fisher's exact test was used for categorical variables. For association study, logistic regression analysis was used to investigate the relationship among *CXCL12* polymorphisms and HCV-therapy response. These analyses were adjusted by the most important clinical and epidemiological characteristics. We included the SNP with the Enter algorithm (Forced Entry) and the covariables with the Stepwise algorithm (at each step, factors are considered for removal or entry: a p-value for entry and exit of 0.15 and 0.20, respectively). Thus, each logistic regression test was only adjusted by the most significant co-variables associated with each one of the outcome variables, avoiding the over-fitting of the regression. The covariables used were gender, age, body mass index, nadir CD4+ T-cells, cART, HCV genotype, HCV-RNA viral load, liver fibrosis, and *IL28B* rs12980275 polymorphism.

Hardy-Weinberg equilibrium (HWE) for all SNPs was assessed by a Chi-square test, considering equilibrium when $p>0.05$. In addition, pair-wise linkage disequilibrium (LD) analysis was computed to detect the inter-marker relationship using the standardized r-squared values by Haploview 4.2 software (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview).

Table 1 shows the clinical and epidemiological characteristics for all 319 patients on HCV treatment at baseline (before starting anti-HCV therapy). The overall rates of the SVR was 54.9% (175/319), 41.5% (90/217) in GT1/4 patients and 83.2% (84/101) in GT2/3 patients.

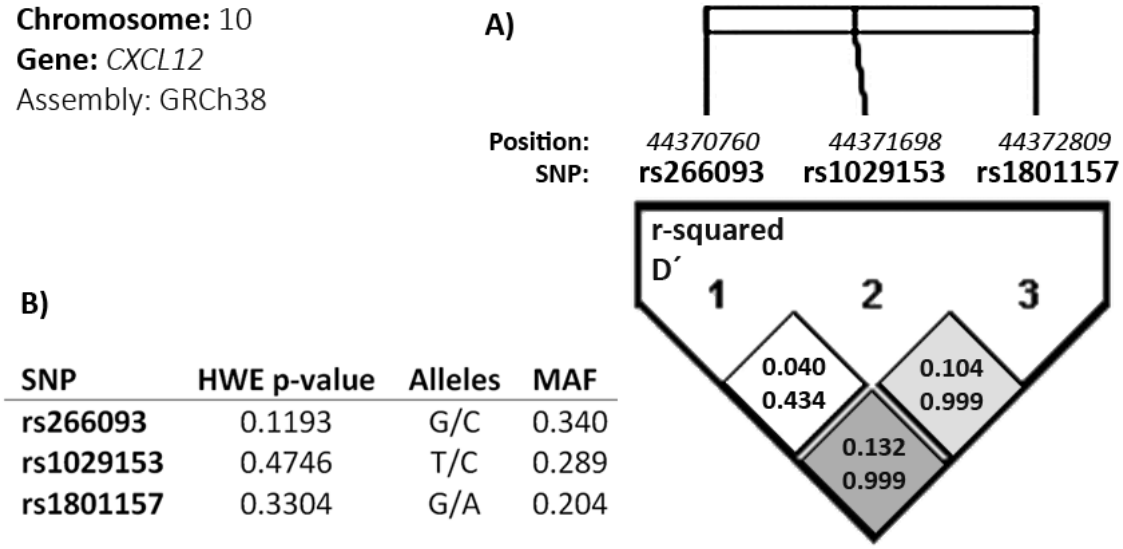
Table 1. Clinical and epidemiological baseline characteristics of HIV/HCV-coinfected patients. Categorical variables are expressed in percentage (absolute count).

	All patients
Number	100% (319)
Gender (male)	77.1% (246)
Age (years)	42 (6.6)
HIV acquired by IVDU	88.1% (281)
Anthropometric values	
Height (m)	1.7 (0.10)
Weight (Kgr)	67 (15)
BMI (kg/m^2)	23.1 (4.2)
BMI ≥25 kg/m^2	28.9% (89)
cART	84% (268)
Current cART protocols	
Any NRTIs + any PI	40.1% (128)
Any NRTIs + PI + NNRTI	0.6% (2)
Any NRTIs + any NNRTI	30.7% (98)
Only NRTIs	8.8% (28)
HIV markers	
Nadir CD4+ T-cells (cells/µL)	226 (208)
Nadir CD4+ <200 cells/µL	44.6% (141)
CD4+ T cells/µL	462 (308)
CD4+ ≥500 cells/µL	44.6% (141)
HIV-RNA <50 copies/mL	66.9% (210)
HCV markers	
HCV-Genotype	
HCV-GT1/4	68.2% (217)
HCV-GT2/3	31.8% (101)
HCV-RNA ≥500,000 UI/ml	73.8% (231)
Liver fibrosis	
Significant fibrosis (F≥2)	63.5% (183)
Advanced fibrosis (F≥3)	34% (98)
IL28B genotype	
rs12980275 (AA)	46.1% (146)

Abbreviations: AIDS, acquired immunodeficiency syndrome; cART, combination antiretroviral therapy; GT, genotype; HCV, hepatitis C virus; HCV-RNA, HCV plasma viral load; HIV, human immunodeficiency virus; HIV-RNA, HIV plasma viral load; IVDU, intravenous drug users; NNRTI, no nucleoside analog reverse-transcriptase inhibitors; NRTI, nucleoside analog reverse-transcriptase inhibitors; PI, protease inhibitors.

Figure 1 shows the characteristics of *CXCL12* SNPs (rs266093, rs1029153 and rs1801157), all located at UTR-3 region. We found a low LD value for the rs266093/rs1029153 couple (D´=0.43), while that LD values were higher for the rs1029153/rs1801157 (D´=0.99) and rs266093/rs1801157 (D´=0.99) (**Figure 1A**). The three SNPs had low r-square values (r-square <0.150), meaning that the *CXCL12*

SNPs did not provide exactly the same information and the *CXCL12* SNPs cannot substitute one for another. Furthermore, all SNPs had a minimum allele frequency (MAF)>20%, displayed missing values <5%, and were in HWE (p>0.05) (**Figure 1B**). The allelic frequencies in our dataset were in accordance with data listed on the NCBI SNP database.



B)

SNP	HWE p-value	Alleles	MAF
rs266093	0.1193	G/C	0.340
rs1029153	0.4746	T/C	0.289
rs1801157	0.3304	G/A	0.204

Figure 1. Characteristics of CXCL12 polymorphisms according to genome assembly GCRh38. (A) Pairwise linkage disequilibrium (LD) patterns for the polymorphisms rs266093, rs1029153 and rs1801157 through CXCL12 gene. Each diagonal represents a different SNP, with each square representing a pairwise comparison between two SNPs. (B) Descriptions of the Hardy-Weinberg equilibrium and alleles frequencies. **Abbreviations:** HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism.

Table 2 shows the relationship between *CXCL12* polymorphism and HCV therapy outcome. Patients with favorable *CXCL12* rs1029153 T allele had higher SVR rate than patients with rs1029153 CC genotype (44% CC, 49% CT, and 61.3% TT; p= 0.025) (**Table 2**). No significant results for rs266093 and rs1801157 polymorphisms were found. In order to define the independent effect of *CXCL12* rs1029153 variants on response to HCV therapy, we performed a multivariate logistic regression adjusted by the most significant variables. Our data showed patients harboring the favorable rs1029153 T allele had significant increased odds for achieving SVR [adjusted odds ratio (aOR)= 1.55 (95% of confidence interval (95%CI)= 1.01; 2.40); p= 0.047)] (**Table 2**). Also, we did not find significant associations for rs266093 and rs1801157 polymorphisms.

Table 2. Summary of the relationship between *CXCL12* polymorphisms (rs266093, rs1029153 and rs1801157) and sustained virologic responses in HIV/HCV-coinfected patients on HCV treatment. Statistically significant differences are shown in bold.

	Unadjusted				Adjusted	
rs266093 G allele	**CC**	**CG**	**GG**	**p-value [(a)]**	**OR (95% CI)**	**p-value [(b)]**
All Patients	57.8% (26/45)	52.1% (75/144)	57.1% (72/126)	0.804	0.94 (0.63; 1.41)	0.777
HCV-GT1/4	48.4% (15/31)	36.8% (35/95)	44.3% (39/88)	0.953	0.81 (0.51; 1.29)	0.337
HCV-GT2/3	78.6% (11/14)	81.6% (40/49)	86.5% (32/37)	0.454	1.63 (0.57; 4.69)	0.363
rs1029153 T allele	**CC**	**CT**	**TT**			
All Patients	44% (11/25)	49.6% (69/139)	61.3% (95/155)	**0.025**	1.55 (1.01; 2.40)	**0.047**
HCV-GT1/4	33.3% (7/21)	37.1% (36/97)	47.5% (47/99)	0.107	1.41 (0.85; 2.35)	0.185
HCV-GT2/3	100% (3/3)	78.6% (33/42)	85.7% (48/56)	0.660	0.83 (0.23; 2.99)	0.770
rs1801157 G allele	**AA**	**AG**	**GG**			
All Patients	50% (4/8)	58.3% (60/103)	52.6% (103/196)	0.487	0.97 (0.57; 1.63)	0.897
HCV-GT1/4	50% (2/4)	44.1% (30/68)	39.4% (54/137)	0.461	1.17 (0.62; 2.21)	0.640
HCV-GT2/3	50% (2/4)	87.7% (30/35)	82.8% (48/58)	0.497	0.94 (0.24; 3.65)	0.931

Abbreviations: 95%CI, 95% of confidence interval; OR, adjusted odds ratio; SVR, sustained virologic response; HCV-GT, hepatitis C virus genotype.
(a), P-values were estimated with Chi-square.
(b), Odds ratios and p-values were calculated by logistic regression analysis adjusted by the most important clinical and epidemiological characteristics (see **statistical analysis** section).

Moreover, no significant association was found when the study population was stratified by HCV genotype (data not shown), possibly due to the low number of patients in each group (217 in HCV-GT1/4 and 101 in HCV-GT2/3). We also performed an analysis between haplotypes of *SDF1* with SVR, but we did not find significant ORs values (data not shown). Finally, we analyzed if there was any epistatic interaction between SDF1 and IL28B polymorphisms, but no significant values were found.

The efficacy of pegIFNα/ribavirin treatment depends, in part, of the interaction of virus and host factors [16]. Among these, the CXCL12/CXCR4 axis also appears to influence the response to pegIFNα/ribavirin therapy since baseline chemotaxis of plamacytoid dendritic cell (pDC) to CXCL12 predicted failure of antiviral response to HCV and correlated with the histological activity index inflammation score [17]. Thus, it could be possible that CXCL12 may be able to recruit inflammatory cells that are not capable to clear HCV during treatment. In this regard, as rs1801157 SNP is located at 3'UTR of *CXCL12* gene, we analyzed *in silico* whether this SNP could be part of microRNAs (miRNAs) binding sites. By using mrSNP, an software to detect SNP effects on microRNA binding [18], we have found that the minor allele generates putative binding sites for hsa-miR-4777-3p/921/1294, whereas the major allele (T) disrupts these target sites and creates one for hsa-miR-3606. Therefore, this SNP could predict to affect the miRNA binding sites, and thus, could be implicate in differences in CXCL12 expression.

CXCR4 is a coreceptor for HIV-1 X4 virus and, as such, plays an important role in virus entry into target cells. Thus, CXCL12 may act as an inhibitor of HIV-1 infection in T-cell CXCR4+ and antiviral agents that bind to CXCR4 may inhibit HIV-1 entry [5]. In this regard, *CXCL12* rs1801157, the best-studied polymorphism in CXCL12 gene, and other polymorphisms in linkage disequilibrium (LD) with the rs1801157 have been associated with higher levels of CXCL12 [19-21]. In HIV infection, *CXCL12* rs1801157 polymorphism has been reported as a strong protection against AIDS progression [10, 11]. Moreover, *CXCL12* rs1801157 polymorphism has also been related to enhanced progression to AIDS and shorter survival [12, 13]. In our study, we found no apparent influence of *CXCL12* SNPs on markers of HIV infection such as nadir CD4+, baseline CD4+, HIV-RNA and prior AIDS diagnosis (data not shown). Likewise, no significant association between polymorphisms and variables related to the natural history of HCV infection (HCV viral load, liver fibrosis, HCV genotype, etc.) was found. However, we cannot rule out a possible role of *SDF1* polymorphisms in the course of HCV natural infection (in non-treated patients). Future studies should be conducted concerning this.

This article focused on pegIFNα/ribavirin treatment. Currently, the new IFNα-free regimens with DAAs have improved the SVR rate, especially for difficult-to treat patients [22]. This fact might diminish the clinical usefulness of biomarkers as predictors of antiviral response. However, not all patients have indications to be treated with these new antivirals. Furthermore, these DAAs treatments are still extremely expensive, which results in serious restrictions for their administration and in the inaccessibility of these drugs in many regions of the world. For these reasons, the pegIFNα/ribavirin still remains as the backbone of some HCV treatment strategies

and the new DAAs are generally being administered in combination with pegIFNα/ribavirin [15]. Thus, we consider that the search for new predictors of pegIFNα/ribavirin-based treatment remains important today and the screening for new polymorphisms is a valid pretreatment approach for maximizing the treatment success and minimizing HCV therapy-related toxicity.

There are some issues that have to be considered for the correct interpretation of the data. Firstly, this report has a retrospective design and the number of patients was relatively small. This could limit the achievement of statistically significant values between *CXCL12* polymorphisms and SVR, especially when we performed the stratified analysis according to the HCV-GT. Secondly, HCV therapy regimens were not identical for all patients since they varied in some characteristics such as pegIFNα 2a or 2b and likely RBV dose. Instead, each physician administered the appropriate HCV therapy regimen according to his/her criteria and by following local and/or international guidelines. Thirdly, the patients selected for our study were patients who met a set of criteria for starting HCV treatment (e.g., no alcohol abuse, high CD4 cell counts, controlled HIV replication, and good treatment adherence), and it is possible that this may have introduced a selection bias. Fourthly, our study was carried out entirely on Caucasians; therefore since the frequency of these alleles varies among ethnicities, an independent study with different ethnic groups would clarify the current data. Fifthly, our study included only HIV/HCV co-infected patients, and may differ in HCV monoinfected patients. However, we did not have access to a cohort of HCV monoinfected patients. The absence of another cohort of mono-infected patients with only HCV may preclude assessing the influence of HIV infection in our results. Sixthly, regarding the statistical significance, there is a considerable controversy about adjusting the "p-value" after multiple tests on clinical-orientated studies [23, 24]. In our study there was a hypothesis supported by theory and previous reports in HIV infected subjects [10-13]. Therefore, we were not literally doing a random search of a meaningful result, and our results should not be affected by the fact of carrying out a high number of statistical tests.

In conclusion, our data show that the presence of favorable *CXCL12* rs1029153 T allele might be related to achieve SVR more easily in HIV/HCV-coinfected patients on pegIFNα/ribavirin therapy. Further analyses are needed to determine its potential use of *CXCL12* rs1029153 polymorphism as a predictive biomarker.

ACKNOWLEDGEMENTS & CONFLICT OF INTEREST DECLARATIONS

Acknowledgements: The authors wish to thank the Spanish National Genotyping Center (CeGen) for providing the SNP genotyping services (http://www.cegen.org).

Funding : This work has been supported by grants given by Fondo de Investigación de Sanidad en España (FIS) [Spanish Health Founds for Research] [grant numbers PI11/01556, PI14/01094, PI11/00245, PI14CIII/00011], Red Española de Investigación en SIDA (RIS) [AIDS Research Network] [grant numbers RD12/0017/0024, RD12/0017/0004 and RD12/0017/0031] and "Fundación para la Investigación y la Prevención del Sida en España" (FIPSE) [grant number 361020/10]. DPT, MAJS, and MGA are supported by "Instituto de Salud Carlos III" [grant numbers CM12/00043, CM10/00105, CD12/00442, respectively]. JB is an investigator from the Programa de Intensificación de la Actividad Investigadora en el Sistema Nacional de Salud (I3SNS).

This work has been (partially) funded by the RD12/0017/00XX project as part of the Plan Nacional R + D + I and cofinanced by ISCIII- Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER).

Competing interest: The authors have nothing to disclose. The authors do not have any financial or personal relationship with other people or organizations that might pose a conflict of interest.

Authors contributions: DPT, MAJS and SR performed all statistical analysis, interpretation of the data and wrote the manuscript. JB and SR participated in the study concept and design. JB, FT, CD, AC, and TAE participated in patient selection, collection of samples and acquisition of data. AFR, SVM, and MGA participated in sample preparation, DNA isolation and genotyping pre-procedure, and contributed with critical revision of the manuscript. SR supervised the study.

All authors revised the manuscript from a draft by SR.

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