Title page

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Title: Analysis of *IL28B* alleles with virologic response patterns and plasma cytokine levels in HIV/HCV coinfected patients

Running head: *IL28B* polymorphisms and anti-HCV therapy

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INTRODUCTION

The current standard therapy in chronic hepatitis C (CHC) consists of pegylated interferon-alpha plus ribavirin (pegIFNα/RBV) [1], which helps to eradicate the virus in nearly 80% of patients infected with HCV genotype 2 or 3, while only about 40-50% of HCV genotype 1 and roughly 60% of genotype 4 patients undergoing therapy clear the virus. Therapy with pegIFNα/RBV is also used as part of combination treatment that includes the new direct acting antivirals Telaprevir or Boceprevir [2]. Human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfected patients show pronounced difficulty at achieving SVR [3]. Moreover, current HCV treatment is prolonged, costly and causes dose-limiting side effects [1]. Thus, the identification of successful predictors of response to therapy is particularly desirable in HIV/HCV coinfected patients in order to ensure an adequate selection of subjects to receive therapy.

HCV treatment response is defined as a reduction or absence of plasma HCV viral load (HCV-RNA), leading to several types of virologic responses, which are designated according to their timing relative to treatment. Thus, viral kinetics is a key factor for determining the optimal duration of therapy that allows for limited exposure to treatment resulting in reduced toxicity and cost savings [1]. Apart from baseline plasma HCV-RNA level, HCV genotype and liver fibrosis are other baseline predictors of response to current HCV therapy [4]. Additionally, several single-nucleotide polymorphisms (SNPs) around the *interleukin 28B* (*IL28B*) gene have been associated with HCV treatment success [5-7]. This gene is clustered with the IFN-λ1 and IFN-λ2 genes on chromosome 19, and encodes IFN-λ3, an interleukin that belongs to the type III Interferon [8]. IFN-λs induce antiviral activity and have been shown to induce interferon-stimulated genes (ISGs) [9]. Beyond the identification of genomic variants, little is yet known about the mechanisms of the association of *IL28B* genotype with HCV clearance. The genetic association between *IL28B* variants and baseline levels of ISGs is a controversial issue, as contradictory results have been observed so far [10].

To date, many articles have assessed the influence of *IL28B* polymorphisms on virologic response in HIV/HCV coinfected patients [11]. The rs8099917 SNP has shown an influence on treatment response among HIV/HCV coinfected patients [12, 13]. However, fewer results have been published regarding the impact on HCV treatment response for other relevant *IL28B* SNPs such as rs12980275, rs7248668, and rs11881222, which are located either in the *IL28B* gene or at its untranslated regions [11, 14]. Those SNPs located in untranslated regions could be involved in the regulation of gene expression and function.

Cytokines are immunomodulation molecules that include interferons (released by host cells in response to the presence of pathogens/tumors) and interleukins (immune response modulators synthesized by helper CD4+ T lymphocytes, monocytes, macrophages, and endothelial cells). The cytokine IL28B is functionally considered an IFN, but it has also been reported to be structurally related to the IL-10 family and the IFN-λ family [8]. Resistance or susceptibility to viral infections such as HCV, is critically linked to cytokine release, which can tend towards different types of responses. Thus, depending on the pattern of signals that CD4+ T cells receive during their initial interaction with pathogens, distinct cell subsets have been reported: Th1, Th2, Th17 and induced regulatory T (iTreg) cells [15]. The cytokine profile plays an important role in treatment response to pegIFNα and ribavirin, and probably modulates the immune response against HCV. Thus, it has been found that a Th2 immune response has been associated with HCV infection persistence [16] and HCV treatment failure [17, 18]. During anti-HCV therapy, IFN-α stimulates Th1 cells but suppresses the Th2 response in HCV-monoinfected patients, suggesting that Th1 rather than Th2 cytokines may be important for eliminating HCV infection [19-21]. Furthermore, Th17 cells produce cytokines that play an important role in autoimmunity and pathogen clearing during host defense reactions [22] making it vital for the clearance of viral infections such as influenza virus [23] and hepatitis B virus [24]. Th17 cells have also been implicated in host defense against HIV [25] and HCV [26]. However, scarce data have been

published on plasma levels of Th17 cytokines in HIV/HCV-coinfected patients on anti-HCV therapy [17].

Hence, our aim was to estimate the impact of *IL28B* polymorphisms (rs12980275, rs8099917, rs7248668, and rs11881222) and to investigate whether their haplotypes could improve HCV treatment success in 326 HIV/HCV-coinfected patients. In addition, we also explored the behavior of plasma cytokine levels in this population.

PATIENTS AND METHODS

Patients

We carried out a retrospective follow-up study on 324 HIV/HCV coinfected patients, who started treatment with pegIFNα/RBV with regular follow-up at two reference HIV hospitals located in Madrid, Spain. All patients were HIV/HCV positive, had completed a course of pegIFNα/RBV therapy, and were genotyped for *IL28B* SNPs. Only IFN-naïve patients were included. Patients with hepatitis B virus (HBV) were excluded. All patients were of European ancestry.

This study was conducted in accordance with the Declaration of Helsinki. Patients gave their written consent for the study and the Institutional Ethics Committee approved it.

Clinical data

The following information was obtained from medical records: age, gender, risk category, weight, height, nadir $CD4^+$ T cell count, antiretroviral therapy, and HCV genotype. In addition, during HCV therapy, blood samples were taken from each patient to analyze complete blood counts, $CD4^+$ T-cells, plasma HIV viral load (HIV-RNA) and HCV-RNA. Liver fibrosis was assessed by different methods depending on the Hospital: a) HGUGM protocol has been described previously [27]. Briefly, the fibrosis score was estimated following the criteria established by the METAVIR Cooperative Study Group [28]: 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. b) HCIII used transient elastometry (FibroScan®, Echosens) as has also been described previously [29]. Briefly, liver stiffness values ≤7.0, between 7.1 and 9.4, and between 9.5 and 12.4, and ≥12.5 were considered to correspond with Metavir scores F0-F1, F2, F3, and F4, respectively [30].

Plasma markers analyzed

Baseline plasma levels of 13 cytokines were assessed (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17A, IL-22 and TNF-α) in a subpopulation of 57 patients, by using Human Th1/Th2/Th9/Th17/Th22 13plex FlowCytomix Multiplex ™ (Bender MedSystems, Vienna, Austria) and *FACSCalibur* flow cytometer (BD *FACSCalibur*™, BD Biosciences, California USA) according to the manufacturer's instructions.

DNA Genotyping

Four *IL28B* single nucleotide polymorphisms (rs12980275, rs8099917, rs7248668, and rs11881222) were genotyped at the Spanish National Genotyping Centre (CeGen; http://www.cegen.org/). Genomic DNA was extracted from peripheral blood with Qiagen columns (QIAamp DNA Blood Midi/Maxi; Qiagen, Hilden, Germany). Genotyping was performed by GoldenGate® assay with VeraCode® Technology (Illumina Inc. San Diego, CA, USA) following manufacturer protocol.

Hepatitis C therapy

HCV treatment regimens included pegIFNα 2a or 2b at standard doses (180 μg/week or 1.5 μg/kg/week, respectively) plus weight-adjusted RBV dosing (1000 mg/day for patients weighing <75 kg and 1200 mg/day for patients weighing ≥75 kg). Following international guidelines [31], patients with HCV genotypes 1 or 4 (GT1/4) received either 48 or 72 weeks of treatment, and patients with HCV genotype 3 (GT3) were treated for 24 or 48 weeks, depending on the virologic response at week 4. None of the patients in this series were infected with HCV genotype 2. Early stopping rules were applied to subjects with suboptimal virologic responses at weeks 12 and 24 [31].

Outcome variables

HCV-RNA viral load was measured by quantitative polymerase chain reaction (qPCR) (Cobas Amplicor HCV Monitor Test, Branchburg, NJ, USA; and COBAS AmpliPrep/COBAS TaqMan HCV test); results were reported in International Units per milliliter (IU/mL), with a lower limit of detection of 10 IU/mL.

Virologic response to HCV treatment was measured by assessing plasma HCV viral load at different time points [1]: a) rapid virologic response (RVR): no detectable viral load (<10 IU/mL) after 4 weeks of treatment; b) early virologic response (EVR): viral load dropped by 99% (2 $\log_{10}$) after 12 weeks of treatment; c) end-of-treatment virologic response (ETVR): no detectable viral load at completion of treatment; d) sustained virologic response (SVR): no detectable viral load six months after treatment cessation.

Statistics

Statistical analysis was carried out by on-treatment (OT) 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.

We performed multivariate logistic regression analysis to investigate the association between favorable *IL28B* genotypes and HCV treatment responses. A stepwise algorithm was used to identify the most significant covariates associated with treatment outcome in our data for the regression model. Hosmer and Lemeshow (H-L) test was performed in order to evaluate the Goodness-of-fit of the regression logistic model. Analysis was performed by using the Statistical Package for the Social Sciences (SPSS) 15.0 (SPSS INC, Chicago, IL, USA).

The Hardy-Weinberg equilibrium (HWE) of all SNPs was assessed by a Chi-square test, considering equilibrium when p value was >0.001 [32]. In addition, haplotype structure was analyzed. Pair-wise linkage disequilibrium (LD) analysis was computed using the standardized D' and r^2 values by Haploview software [33]. Only those haplotypes with a frequency >0.01 were studied. Each haplotype was analyzed versus all others by a multivariate logistic regression analysis adjusted by the most significant covariates cited above. Therefore, odds ratios (OR) and p-values of each comparison were calculated in respect to the overall data. These analyses were performed with PLINK software [34].

We also carried out a classification and regression tree (CART) to identify higher-order interactions between SVR and *IL28B* variants using SPSS 15.0. CART is a prognostic system with a hierarchical structure based on recursive portioning that build a decision tree to identify subgroups at higher odds of SVR. CART was performed with SPSS 15.0.

In regard to cytokine levels, data were analyzed according to *IL28B* genotypes by the Mann-Whitney U test, and p-values were adjusted by Bonferroni correction. All p-values were two-tailed and statistical significance was defined as $p<0.05$. This analysis was also carried out by SPSS 15.0.

RESULTS

Patients

Our study included 324 HIV/HCV coinfected patients on HCV treatment, whose baseline characteristics (before starting anti-HCV therapy) are shown in **Table 1**. A total of 184 out of 324 patients, (56.8%) were GT1, 103 (31.6%) were GT3, and 37 (11.4%) were GT4. We did not find significant differences between patients according to HCV genotype (**Table 1**).

Table 1. Main baseline epidemiological and clinical characteristics of HIV/HCV coinfected patients on HCV antiviral therapy.

	All patietns	**HCV GT1**	**HCV GT3**	**HCV GT4**
No.	324	184	103	37
Male *	249 (76.9%)	151 (82.1%)	71 (68.9%)	27 (73%)
Age (years) †	41.9 (38.6 – 45.2)	41.0 (38.1 – 45.1)	42.7 (39.4 – 46.0)	43.0 (39.0 – 45.1)
IVDU *	284 (87.7%)	156 (84.8%)	93 (90.3%)	35 (94.6%)
HAART *	274 (84.6%)	154 (93.7%)	85 (82.5%)	35 (94.6%)
Anthropometric values				
Height (m) †	1.70 (1.65 – 1.75)	1.71 (1.66 – 1.75)	1.69 (1.64 – 1.74)	1.70 (1.65 – 1.74)
Weight (Kgr) †	67 (60 – 76)	68 (62 – 76)	64 (59 – 73)	65 (62 – 75)
BMI (kg/m^2) †	23.1 (21.2 – 25.4)	23.5 (21.4 – 25.6)	22.7 (20.6 – 24.6)	22.9 (21.3 – 20.0)
BMI ≥25 kg/m^2	92 (29.6%)	61 (33.9%)	20 (20.8%)	11 (31.4%)
HIV markers				
Nadir CD4+ T-cells/µL †	224 (131 - 330)			
Nadir CD4+ <200 cells/µL *	141 (43.5%)	79 (42.9%)	46 (44.7%)	16 (43.2%)
Baseline CD4+ T-cells/µL †	468 (372 - 672)	222 (133 - 333)	224 (131 - 355)	228 (125 - 314)
CD4+ ≥500 cells/µL *	145 (45.2%)	86 (47%)	40 (39.6%)	19 (51.4%)
HIV-RNA <50 copies/mL *	212 (65.4%)	114 (62.6%)	71 (71%)	27 (73%)
HCV markers *				
Baseline HCV-RNA ≥500,000 IU/mL	235 (73.3%)	139 (76.4%)	72 (72%)	24 (64.9%)
Liver fibrosis (n= 286)*				
Significant fibrosis (F≥2)	182 (63.6%)	105 (64.8%)	58 (65.2%)	19 (54.3%)
Advanced fibrosis (F≥3)	98 (34.3%)	51 (31.5%)	33 (37.1%)	14 (40%)
Cirrhosis (F4)	50 (17.5%)	23 (14.2%)	21 (23.6%)	6 (17.1%)

(*), Absolute number (percentage); (†), Median (25th – 75th percentiles). Sometimes, the percentages were not calculated from all patients because some data was missing.
Abbreviations: BMI, body mass index; IVDU, intravenous drug users; HAART: highly active antiretroviral therapy; HCV: Hepatitis C virus; HCV-RNA: HCV plasma viral load; HIV: Human immunodeficiency virus; HIV-RNA: HIV plasma viral load.

The overall rates of the four efficacy endpoints (RVR, EVR, ETVR, and SVR) were 33.2%, 72.3%, 64.2% and 54.6%, respectively (see **Supplemental Digital Content (SDC) 1**). When we stratified these data by HCV genotype, different rates of HCV treatment response were found for the four efficacy endpoints, with the highest response rates for GT3 patients (58.9%, 92.5%, 88.1% and 82.5% for RVR, EVR, ETVR and SVR, respectively), while lower response rates were observed for GT1 and GT4 patients (see **SDC1**).

Linkage disequilibrium among IL28B SNPs

We analyzed four *IL28B* SNPs (rs12980275, rs11881222, rs8099917, and rs7248668). All of them showed a call rate of 99.53%, fulfilled the minimum allele frequency (MAF)>0.05, and were in HWE (p>0.001). Linkage disequilibrium (LD) was identified among the four *IL28B* SNPs, especially between rs12980275/rs11881222 (D´=0.97; r^2=0.94) and rs8099917/rs7248668 (D´=1.00; r^2=0.99) (see **SDC2)**).

IL28B variants and virologic response to HCV treatment

Due to the high LD among the cited *IL28B* polymorphisms, we obtained the same or highly similar results for rs12980275/rs11881222 and rs8099917/rs7248668 couples. In order to avoid redundancy, we only show results for rs12980275 and rs8099917.

As is shown in **Figure 1**, interestingly the rate of virologic response at different time points (RVR, EVR, ETVR and SVR) was significantly higher in subjects harboring favorable *IL28B* genotypes (rs12980275 (AA) and rs8099917 (TT)). These differences were mainly observed for genotype 1 **(Figure 1)**. All numerical data are provided in **SDC3**.

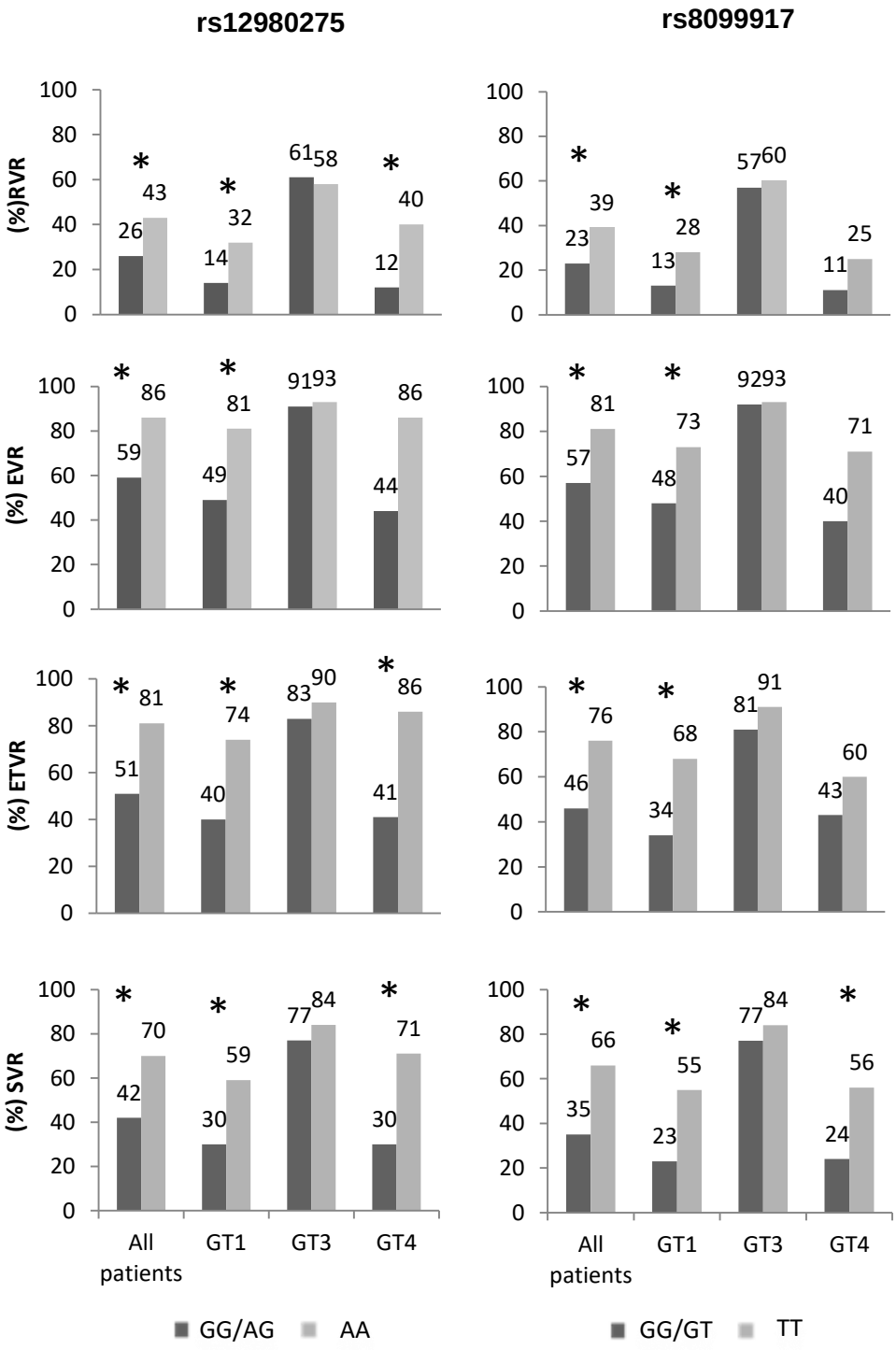

Figure 1. Percentage of HIV/HCV coinfected patients showing different virologic responses to HCV treatment varies depending on *IL28B* genotypes. Abbreviations: GT1/4, Hepatitis C virus genotype 1/4; GT3, Hepatitis C virus genotype 3, RVR, rapid virologic response; EVR, early virologic response; ETR, end-of-treatment virologic response; SVR, sustained virologic response.

In order to analyze the effect of *IL28B* variants on virologic response, we performed a multivariate logistic regression adjusted by the most significant variables, which were selected by stepwise algorithm (HCV genotype, HCV-RNA ≥500,000 IU/Ml and significant fibrosis (F≥2)). The Hosmer-Lemeshow goodness-of-fit test showed no evidence of lack of fit (**SDC4**). The dominant model was the best fit to our data. The association of *IL28B* variants with virologic response during HCV

treatment (RVR, EVR, and ETVR) and after completion of HCV therapy (SVR) was statistically significant for all patients and for GT1/4 patients (**Table 2**). It is noteworthy that patients harboring favorable *IL28B* genotypes (either rs12980275 (AA) or rs8099917 (TT)) showed, in some cases, virologic response rates around four times higher than those patients with unfavorable genotypes ($p<0.001$) (**Table 2**). When patients were stratified by HCV genotype, the association was observed for GT1/4 patients, and for GT1 and GT4 patients, separately. Overall, both *IL28B* SNPs showed similar results. Regarding HCV genotype, the results for GT4 patients are less conclusive, because the low number of patients (n= 37) prevents us from drawing firm conclusions (**Table 2**). For HCV GT3 patients, we did not find any significant association (data not shown).

Table 2. Adjusted odds of achieving virologic responses according to *IL28B* genotypes in HIV/HCV coinfected patients on HCV treatment.

	RVR		EVR		ETVR		SVR	
	OR(95% CI)	**p**	**OR(95% CI)**	**p**	**OR(95% CI)**	**p**	**OR(95% CI)**	**p**
Overall [(a)]								
rs12980275 (AA)	1.62 (0.82 – 3.22)	0.165	4.59 (2.25 – 9.34)	**<0.001**	3.47 (1.93 – 6.23)	**<0.001**	2.86 (1.67 – 4.97)	**<0.001**
rs8099917 (TT)	1.61 (0.79 – 3.29)	0.189	2.67 (1.45 – 4.91)	**0.002**	2.72 (1.56 – 4.73)	**<0.001**	3.20 (1.82 – 5.64)	**<0.001**
HCV GT1/4 [(b)]								
rs12980275 (AA)	3.28 (1.31 – 8.20)	**0.011**	5.86 (2.65 – 12.9)	**<0.001**	4.12 (2.14 – 7.91)	**<0.001**	3.66 (1.93 – 6.94)	**<0.001**
rs8099917 (TT)	2.70 (1.03 – 7.07)	**0.043**	3.01 (1.56 – 5.79)	**0.001**	2.73 (1.49 – 5.01)	**0.001**	3.56 (1.86 – 6.79)	**<0.001**
HCV GT1 [(b)]								
rs12980275 (AA)	2.04 (0.69 – 5.94)	0.192	5.19 (2.19 – 12.2)	**<0.001**	3.41 (1.69 – 6.87)	**0.001**	3.03 (1.50 – 6.12)	**0.002**
rs8099917 (TT)	2.24 (0.71 – 7.13)	0.170	2.67 (1.34 – 5.29)	**0.005**	2.67 (1.34 – 5.29)	**0.005**	3.07 (1.48 – 6.39)	**0.003**
HCV GT4 [(b)]								
rs12980275 (AA)	6.65 (0.61 – 72.4)	0.120	13.1 (1.13 – 151.1)	**0.039**	24.7 (1.82 – 334.8)	**0.016**	7.46 (1.01 – 55.1)	**0.049**
rs8099917 (TT)	1.48 (0.17 – 13.1)	0.722	4.27 (0.82 – 22.1)	0.083	3.02 (0.61 – 14.9)	0.175	4.32 (0.86 – 21.7)	0.075

Statistically significant differences are shown in bold.

(a), These tests were adjusted by HCV-genotype 1 or 4 versus 3, HCV-RNA ≥500,000 IU/mL and significant fibrosis (F≥2). .

(b), These tests were adjusted by HCV-RNA ≥500,000 IU/ml and significant fibrosis (F≥2).

Abbreviations: RVR, rapid virologic response; EVR, early virologic response; ETVR, end-of-treatment virologic response; SVR, sustained virologic response; OR, odds ratio; 95% CI, 95% confidence interval; HCV, Hepatitis C virus.

Next, we examined whether the combination of all four *IL28B* SNPs could improve HCV treatment outcome. Thus, any association of *IL28B* haplotypes defined by all four SNPs was assessed. The haplotype AATG, which harbors all favorable alleles, was the most prevalent (67%), followed by GGGA (19%) with all non-protective alleles, and GGTG (12%) possessing two protective alleles (**Table 3**). Therefore, patients harboring AATG showed increased OR of achieving EVR, ETVR and SVR, while GGGA showed decreased OR for all variables. The haplotype GGTG, which contains two protective alleles, was only significant for EVR and ETVR (OR= 0.50, p=0.023 and OR=0.56, p=0.045, respectively). When patients were analyzed by HCV genotype, we found that only significant differences were found for GT1 and 4 patients, where ORs were higher for favorable haplotype (AATG) and more reduced for unfavorable haplotype (GGGA) compared with the overall analysis (**Table 3**). For GT3 patients, we did not find any significant association (data not shown).

Table 3. Adjusted odds of achieving virologic responses according to *IL28B* haplotypes formed by rs12980275, rs11881222, rs8099917, rs7248668.

		RVR		EVR		ETVR		SVR	
	Freq	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
Overall [(a)]									
AATG	0.67	1.55 (0.92-2.64)	0.098	2.86 (1.76-4.67)	**<0.001**	2.73 (1.75-4.26)	**<0.001**	2.47 (1.59-3.83)	**<0.001**
GGTG	0.12	0.68 (0.31-1.49)	0.330	0.50 (0.27-0.91)	**0.023**	0.56 (0.32-1.00)	**0.047**	0.80 (0.45-1.42)	0.451
GGGA	0.19	0.72 (0.38-1.36)	0.307	0.48 (0.28-0.81)	**0.006**	0.44 (0.27-0.73)	**0.001**	0.40 (0.24-0.67)	**<0.001**
HCV GT1/4 [(b)]									
AATG	0.63	2.75 (1.25-6.05)	**0.007**	3.13 (1.82-5.39)	**<0.001**	3.09 (1.86-5.15)	**<0.001**	3.04 (1.80-5.13)	**<0.001**
GGTG	0.13	0.52 (0.17-1.53)	0.206	0.53 (0.28- 1.01)	**0.050**	0.53 (0.28-0.99)	**0.040**	0.74 (0.39-1.39)	0.338
GGGA	0.22	0.42 (0.16-1.08)	0.061	0.39 (0.21- 0.70)	**0.001**	0.39 (0.22-0.69)	**<0.001**	0.31 (0.17-0.58)	**<0.001**
HCV GT1 [(b)]									
AATG	0.65	2.07 (0.88-4.86)	0.085	3.10 (1.71-5.63)	**<0.001**	2.73 (1.59-4.68)	**<0.001**	2.71 (1.54-4.76)	**<0.001**
GGTG	0.13	0.69 (0.21-2.26)	0.534	0.46 (0.23-0.95)	**0.035**	0.59 (0.30-1.18)	0.130	0.73 (0.36-1.50)	0.383
GGGA	0.20	0.45 (0.14-1.41)	0.158	0.41 (0.20-0.80)	**0.009**	0.37 (0.19-0.72)	**0.002**	0.33 (0.16-0.67)	**0.001**
HCV GT4 [(b)]									
AATG	0.54	6.44 (0.69-60)	0.084	4.44 (0.91-21.8)	**0.045**	14.29 (1.84-111)	**0.002**	4.15 (0.84-20.6)	0.059
GGTG	0.15	0	0.114	0.94 (0.22-3.93)	0.929	0.30 (0.05-1.78)	0.147	0.82 (0.19-3.59)	0.786
GGGA	0.31	0.61 (0.08-4.42)	0.622	0.28 (0.07-1.18)	0.066	0.36 (0.09-1.46)	0.137	0.36 (0.09-1.43)	0.130

Statistically significant differences are shown in bold.

([a]), These tests were adjusted by HCV-genotype 1 or 4 versus 3, HCV-RNA ≥500,000 IU/mL and significant fibrosis (F≥2). .

([b]), These tests were adjusted by HCV-RNA ≥500,000 IU/ml and significant fibrosis (F≥2).

Abbreviations: RVR, rapid virologic response; EVR, early virologic response; ETVR, end-of-treatment virologic response; SVR, sustained virologic response; OR, odds ratio; 95% CI, 95% confidence interval, HCV, Hepatitis C virus.

Finally, we explored, by a decision tree algorithm, whether two IL28B SNPs may predict SVR better than one single SNP in those patients with HCV GT1/4, where IL28B SNPs are associated with virologic response rates. For this purpose we used genotype data from rs12980275 and rs8099917 polymorphisms. The overall SVR rate of 41.9% significantly increased to 59.1% in those patients carrying the rs12980275 favorable genotype (AA), but this rate did not improve when considering patients simultaneously carrying protective genotypes for both SNPs **(Figure 2).** Moreover, patients carrying the rs12980275 unfavorable genotypes (AG/GG) showed an increase in SVR rate (from 30.6% to 45.2%) when they also possessed the rs8099917 favorable genotype (AA). On the other hand, SVR decreased from 30.6% to 23.9% when one of the rs8099917 unfavorable genotypes (GT/GG) was present in these patients **(Figure 2).**

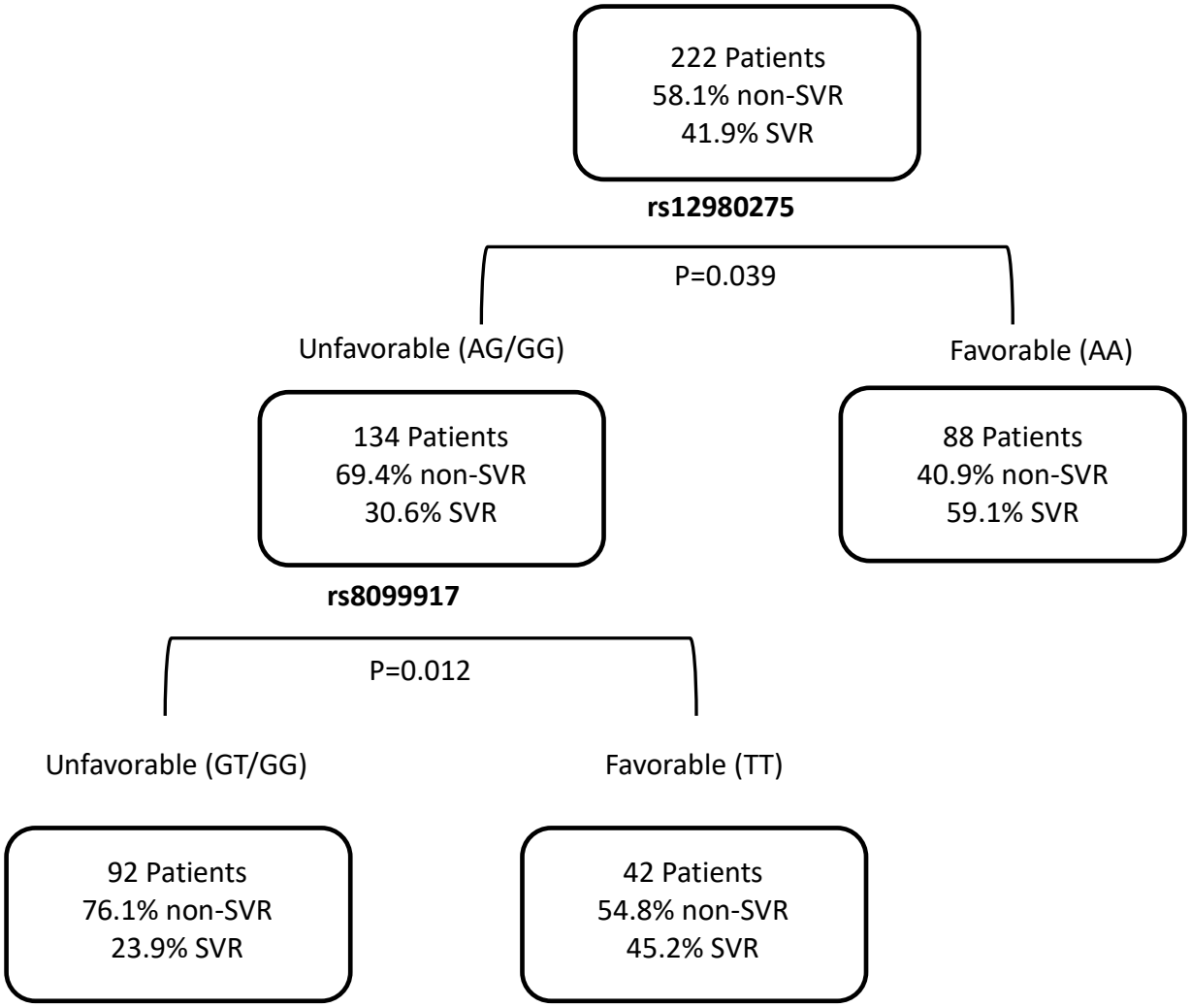

Figure 2. Decision tree representation of HIV/HCV genotype 1/4 coinfected patients for SVR. Nodes have been stratified by *IL28B* genotypes for rs12980275 and rs8099917 polymorphisms. Only significant nodes are shown.

Influence of IL28B genotype on cytokines level and SVR

We analyzed baseline plasma levels of Th1/Th2/Th9/Th17/Th22 cytokines (13plex) and their relevance to *IL28B* genotypes in the 57 out of 324 patients for whom baseline plasma samples were available. Unfortunately, we were unable to analyze the effect of rs8099917 due to the unequal distribution of patients in each group (one of the groups displayed only one patient). Hence, patients

were classified according to their favorable or unfavorable rs12980275 genotype. Only data for cytokines significantly associated with other variables are shown (IFN-γ, IL-5, IL-9, TNF-α, and IL-6).

First, patients were stratified by *IL28B* rs12980275 genotype (see **SDC5**). Subjects harboring the favorable genotype (AA) showed reduced cytokine levels in general, with the difference being significant for IFN-γ, IL-5 and IL-9. After adding a second factor of stratification (HCV genotype) (see **SDC6**), we found that subjects with the best prognosis in terms of treatment response (favorable *IL28B* genotype (AA) and HCV genotype 3) had significantly reduced IFN-γ and IL-9 plasma levels compared to those less likely to respond to treatment (*IL28B* genotype (GG+AG) and HCV genotype 1/4). Finally, patients were stratified according to rs12980275 genotype and SVR (**Figure 3**). Again, the most interesting results were found when comparing the extreme groups. Non-responder patients with unfavorable *IL28B* genotype (no-SVR, AG/GG) had significantly higher values of IFN-γ, IL-6, IL-9, and TNF-α than responder patients with favorable *IL28B* genotype (SVR, AA) (**Figure 3**).

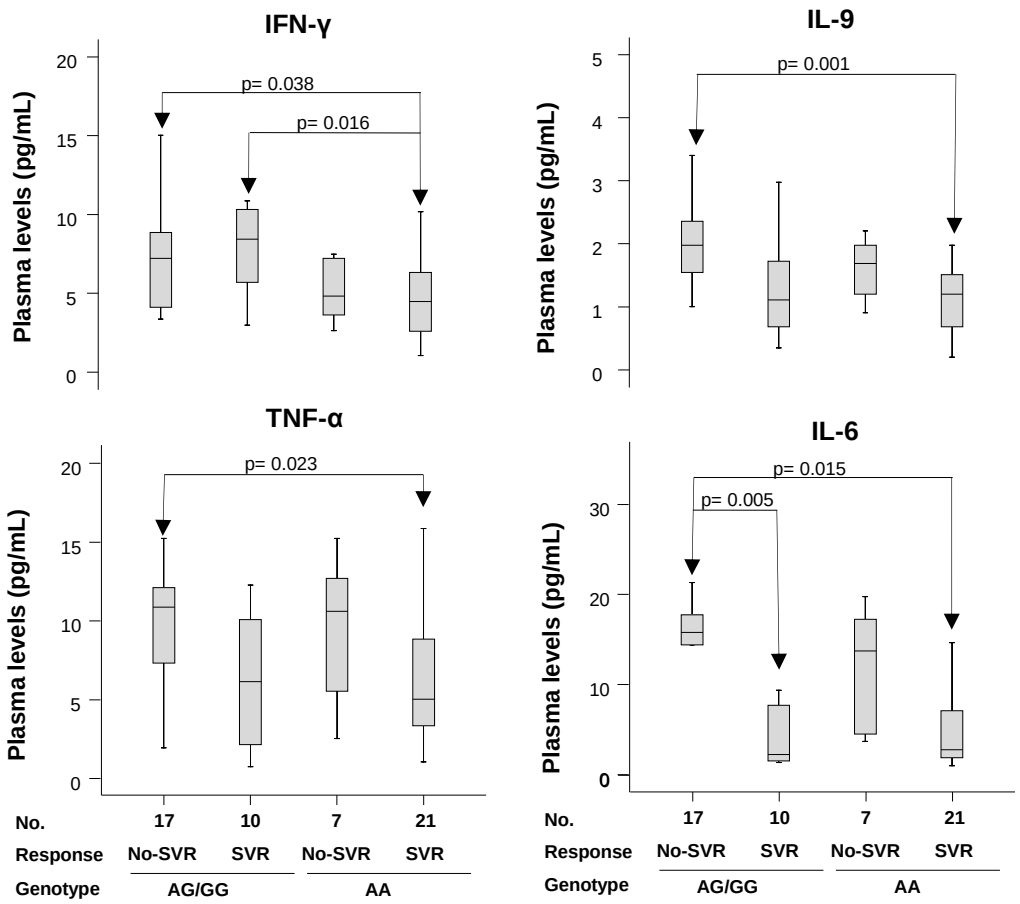

Figure 3. Cytokine levels of HIV/HCV coinfected patients at pretreatment, according to rs12980275 genotype and sustained virologic response (SVR)

We performed a multiple linear regression to assess the influence of these three factors together (SVR, *IL28B* genotype, and HCV genotype) on cytokine levels ($logarithm_{10}$ transformed, dependent variable). The slope (b) of the regression equation (y=a + b*X) allows us to quantify the effect of each variable on cytokine levels. We found that IFN-γ levels were lower in rs12980275 (AA) than in rs12980275 (AG/GG) patients, (b=-0.257±0.081; p=0.003), but this cytokine was not influenced by HCV genotype or SVR. However, IL-6 (b=-0.380±0.146; p=0.012), IL-9 (b=-0.293±0.102; p=0.006), and TNF-α (b=-0.218±0.099; p=0.032) levels were all lower in SVR than in no-responder patients, but these cytokines, in contrast, were not influenced by *IL28B* or HCV genotype.

DISCUSSION

In the present study, we have selected four seldom-studied *IL28B* SNPs for their assessment in HIV/HCV coinfected patients. Our results showed a significant association of all studied SNPs alone (rs12980275, rs11881222, rs8099917, and rs7248668) or in combination (*IL28B* haplotypes) with HCV-treatment response in HCV/HIV coinfected patients.

It is noteworthy that this is the first study reporting treatment response data on rs11881222 and rs7248668 in HIV/HCV coinfected patients. To our knowledge only one study on this topic reported data from Caucasian HCV mono-infected patients [35], as it has mainly been studied in Asian populations [7, 36, 37]. Clausen et al. [38] also reported data for rs11881222 in HIV/HCV coinfected patients, but their results are not comparable to our study, as they analyzed the association with a different variable (spontaneous clearance), and some of the patients were also coinfected with HBV. In addition, we have shown for the first time the association of seldom-studied *IL28B* SNPs with all virologic responses during and after HCV therapy (RVR, EVR, ETVR SVR) in HIV/HCV coinfected patients. Although results for all four of the SNPs were highly similar, it seems that rs12980275 shows higher association with on-treatment responses (EVR and ETVR). In addition to being commonly used, these data on virologic responses are important for the monitoring of patients and the identification of non-responders and relapses. For example, EVR is a pivotal decision criterion for treatment guidelines, since it has been shown that the absence of EVR is the most robust characteristic for identifying non-responders [1].

In addition to host factors, HCV genotype is also a well-known predictor of response to pegIFNα/RBV therapy [1], as patients with GT1/4 show poorer responses (40-50%) than GT2/3 (80%) [39]. About two thirds of our cohort had GT1/4 viruses, and we confirmed the strong association of *IL28B* polymorphisms with the rate of on-treatment virologic responses for both groups. Results for GT1 and GT4 were similar, although GT4 patients showed less significant results than GT1. This lack of consistency in GT4 patients is probably due to the limited sample size. On the other hand, the genotyping of *IL28B* SNPs was useless for predicting therapy outcome in HIV/HCV patients infected with GT3. This attenuated association for HCV GT3 patients could be related to the high rate of SVR present with these IFN-sensitive genotypes, where a larger sample size would be required to tease out statistical differences [40].

IL28B haplotypes were investigated in order to evaluate whether the association with treatment outcome was better explained using this approach. AATG (four favorable alleles) was the most prevalent and highly significant haplotype for predicting all virologic responses (RVR, EVR, ETVR and SVR). However in contrast, GGGA (four non-favorable alleles) showed decreased odds for all virologic response, and was therefore an excellent predictor of HCV treatment failure. Nevertheless, as has been previously reported for spontaneous HCV clearance [41], haplotype analysis did not improve the prediction of any of the virologic responses compared with the SNPs alone.

However, the use of a decision tree tool appeared to improve treatment success prediction. Thus, it was found that HCV GT1/4 patients with an unfavorable rs12980275 genotype significantly improved the prediction of SVR when they were combined with the rs8099917 genotype information. Therefore, the genotyping of only rs12980275 may be sufficient for predicting treatment response in patients with a favorable genotype (AA), while for subjects harboring unfavorable rs12980275 (AG or GG), genotyping of rs8099917 is advisable. This promising result will allow us to identify subjects with a very low likelihood of responding to pegIFNα/RBV treatment prior to prescribing it, which is extremely important in order to avoid unnecessary side effects of an ineffective treatment [1]. In this context, patients with a double-poor-responder genotype should be treated with new direct-acting antivirals (DAAs), such as boceprevir and telaprevir, instead of pegIFNα/RBV alone or wait until additional new DAAs become available. Since HIV/HCV coinfected patients have generally poorer responses compared with HCV monoinfected individuals [42, 43], HCV therapy is particularly not

recommended for coinfected patients with a low likelihood of achieving a virologic response, and screening for *IL28B* SNPs is a valid pre-treatment approach for maximizing treatment success and minimizing HCV therapy-related toxicity.

Both viral and host factors are independent predictors of HCV treatment outcome [3], nonetheless both have an effect on the immune response. To further investigate this, we studied whether *IL28B* variants (rs12980275) affect baseline levels of different cytokines related to different types of immune responses. In order to achieve this goal, patients were also stratified by HCV genotype and SVR. In our study, patients with the poorest prognosis (AG or GG at rs12980275 and HCV GT1/4) were those who showed the highest values of Th1 (IFN-γ) and Th2 (IL-9) cytokines. Likewise, patients with unfavorable rs12980275 genotype (AG or GG) that did not achieve SVR, showed the highest values of Th1 (IFN-γ), Th2 (IL-6 and IL-9), and pro-inflammatory (TNF-α) cytokines before starting anti-HCV treatment.

The rs12980275 SNP seems to be located at the promoter region of the *IL28B* gene, therefore it could modify its expression and the expression of related interferon-stimulated genes (ISGs) [44]. ISGs play a critical role in anti-HCV therapy response [45]. It is tempting to speculate that patients with the favorable genetic variation of this SNP may have a more effective immune response to HCV infection, which therefore results in a better response to anti-HCV treatment.

IFN-γ is secreted by cytotoxic T lymphocytes (CTLs) and plays a pivotal role in mechanisms of HCV control [46]. HCV infection induces the endogenous IFN system in the liver of some (but not all) patients with CHC; however, patients with a pre-activated IFN system are refractory to both endogenous and exogenous IFN-α [47]. In our study, we found that patients with non-favorable *IL28B* genotypes and HCV GT1/4 who do not achieve SVR showed the highest values of IFN-γ, which may indicate a greater degree of the pre-activated IFN system associated to HCV treatment failure. In this sense, our results agree with previous studies that have detected an increase in plasma levels of the IFN-γ inducible protein 10 (IP-10) (CXCL10) in CHC non-responder patients with HCV genotype 1 [47-49]. These high CXCL10 levels may also indicate a pre-activation of the IFN system in the liver [47].

TNF-α is a pro-inflammatory cytokine released by different types of cells, and it has been implicated in non-response to interferon therapy in CHC [50]. In our study, high levels of TNF-α were found in patients with non-favorable *IL28B* genotype and without SVR. TNF-α may influence the response to anti-HCV therapy via over-expression of suppressors of cytokine signaling 3 (SOCS3) in the livers of CHC patients [51, 52].

The Th2 immune response has been associated with HCV treatment failure in HIV/HCV coinfected patients [17] and HCV monoinfected patients [18]. Our data showed that patients achieving SVR displayed reduced levels of Th2 cytokines (IL-9 and IL-6), but interestingly, a different expression pattern was found for each cytokine. Plasma levels of IL-9 seemed to be dependent on *IL28B* genotype and HCV genotype, while IL-6 did not. As for TNF-α, it is possible that IL-6 and IL-9 may influence the response to anti-HCV therapy via over-expression of SOCS3, a potent inhibitor of the type I IFN pathway, by producing IFN-α resistance through suppression of interferon signaling [53-55].

This study has several limitations that must be taken into account for the correct interpretation of the data. Our study design is retrospective and has a low number of patients. All selected patients 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 this may have introduced a selection bias. Since p-value is dependent on the sample size, it may be possible that we did not find any significant adjusted p-values in some comparisons (GT4, for instance) due to such a size-limited population. Thus, only big effects would be detected in small populations. Finally, as with any study measuring plasma levels of cytokines, any additional unknown infection(s) could potentially influence

our results, although we have no evidence. With this in mind, we confirmed the absence of hepatitis B virus infection or other active opportunistic infections in all patients.

In conclusion, the four *IL28B* polymorphisms tested alone or by haplotypes showed a significant association with HCV clearance during and after HCV therapy. Although haplotypes did not improve the prediction of SVR, the decision tree with rs12980275 and rs8099917 was useful for improving the prediction of HCV treatment success in the most difficult-to-treat patients, for whom adding more factors to predictive algorithms could improve therapeutic decision-making. Furthermore, the cytokine profile was much more favorable in patients with rs12980275 (AA) who achieved SVR.

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AUTHORS CONTRIBUTIONS

AFR and SR performed all statistical analysis, interpretation of the data and wrote the manuscript.

JB, VS, and SR participated in the study concept and design. JB, VS, JC, JCL, and PM participated in patient selection, collection of samples and acquisition of data. MAJS, MGF, MGA, JMB, CR, and NR 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 AFR.

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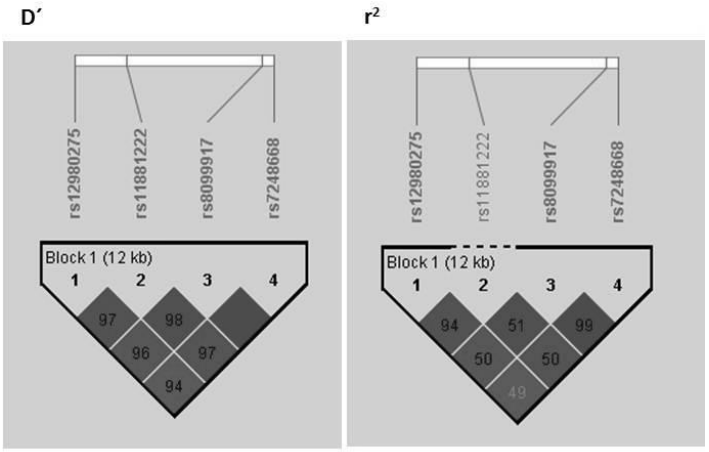
SUPPLEMENTAL DIGITAL CONTENT (SDC)

SDC 1: Number of patients from each HCV genotype that achieve each efficacy endpoint and the pattern of virologic response.

	Nº (%)	RVR	EVR	ETVR	SVR
All patients	324 (100%)	75/226 (33.2%)	209/289 (72.3%)	204/318 (64.2%)	177/324 (54.6%)
HCV GT1/4	221 (68.2%)	32/153 (20.9%)	122/195 (62.5%)	115/217 (52.9%)	92/221 (41.6%)
HCV GT1	184 (56.8%)	27/123 (21.9%)	104/161 (64.6%)	98/181 (54.1%)	78/184 (42.4%)
HCV GT4	37 (11.4%)	5/30 (16.7)	18/34 (52.9%)	17/36 (47.2%)	14/37 (37.8%)
HCV GT3	103 (31.6%)	43/73 (58.9%)	87/94 (92.6%)	89/101 (88.1%)	85/103 (82.5%)

Abbreviations: RVR, rapid virologic response; EVR, early virologic response; ETVR, end-of-treatment virologic response; SVR, sustained virologic response; HCV, Hepatitis C virus.

SDC2. Pairwise linkage disequilibrium (LD) patterns for four polymorphisms through IL28B regions. Each diagonal represents a different SNP, with each square representing a pairwise comparison between two SNPs.



SNPs	D´	LOD	r^2
rs12980275/rs11881222	0.977	110.09	0.941
rs12980275/rs8099917	0.96	45.66	0.503
rs12980275/rs7248668	0.948	44.47	0.494
rs11881222/rs8099917	0.986	47.37	0.513
rs11881222/ rs7248668	0.973	45.84	0.503
rs8099917/rs7248668	1.0	101.95	0.99

SCD3: Number of patients from each HCV genotype stratified by rs12980275 (a) and rs8099917 (b) that achieve each efficacy endpoint and the pattern of virologic response.

(a) rs12980275

Responders/total (%)

RVR	**All patients**	**GT1**	**GT3**	**GT4**
AG+GG	33/127 (26)	10/69 (14)	20/33 (61)	3/25 (12)
AA	42/98 (43)	17/53 (32)	23/40 (58)	2/5 (40)
EVR				
AG+GG	90/153 (59)	43/88 (49)	29/32 (91)	12/27 (44)
AA	114/132 (86)	57/70 (81)	51/55 (93)	6/7 (86)
ETVR				
AG+GG	87/170 (51)	40/100 (40)	29/35 (83)	12/29 (41)
AA	119/147 (81)	59/80 (74)	54/60 (90)	6/7 (86)
SVR				
AG+GG	73/174 (42)	31/103 (30)	27/35 (77)	3/30 (30)
AA	104/149 (70)	47/80 (59)	52/62 (84)	5/7 (71)

(b) rs8099917

Responders/total (%)

RVR	**All patients**	**GT1**	**GT3**	**GT4**
TG+GG	20/86 (23)	6/47 (13)	12/21 (57)	2/18 (11)
TT	55/140 (39)	21/76 (28)	31/52 (60)	3/12 (25)
EVR				
TG+GG	60/106 (57)	30/62 (48)	22/24 (92)	8/20 (40)
TT	145/180 (81)	71/97 (73)	64/69 (93)	10/14 (71)
ETVR				
TG+GG	54/117 (46)	24/70 (34)	21/26 (81)	9/21 (43)
TT	153/201 (76)	76/111 (68)	68/75 (91)	9/15 (60)
SVR				
TG+GG	42/120 (35)	17/73 (23)	20/26 (77)	5/21 (24)
TT	135/204 (66)	61/111 (55)	65/77 (84)	9/16 (56)

Abbreviations: RVR, rapid virologic response; EVR, early virologic response; ETVR, end-of-treatment virologic response; SVR, sustained virologic response; HCV, Hepatitis C virus.

SDC4: Hosmer and Lemeshow Goodness-of-Fit test for the regression logistic model

	RVR		EVR		ETVR		SVR	
	Chi-square	**Sig.**	**Chi-square**	**Sig.**	**Chi-square**	**Sig.**	**Chi-square**	**Sig.**
Overall [(a)]								
rs12980275 (AA)	9.119	0.244	4.865	0.772	6.700	0.461	8.428	0.296
rs8099917 (TT)	9.946	0.192	5.248	0.630	6.033	0.536	5.526	0.596
HCV GT1/4 [(b)]								
rs12980275 (AA)	5.959	0.814	6.538	0.257	4.361	0.359	2.435	0.656
rs8099917 (TT)	1.540	0.908	5.977	0.426	5.763	0.330	2.418	0.789
HCV GT1 [(b)]								
rs12980275 (AA)	1.827	0.768	6.647	0.355	4.305	0.506	2.458	0.652
rs8099917 (TT)	1.498	0.827	5.238	0.388	5.238	0.388	1.185	0.881
HCV GT4 [(b)]								
rs12980275 (AA)	2.134	0.711	3.153	0.533	2.211	0.697	3.646	0.456
rs8099917 (TT)	2.521	0.773	2.854	0.723	4.751	0.576	4.109	0.391

(a), These tests were adjusted by HCV-genotype 1 or 4 versus 3, HCV-RNA ≥500,000 IU/mL and significant fibrosis (F≥2). .

(b), These tests were adjusted by HCV-RNA ≥500,000 IU/ml and significant fibrosis (F≥2).

Abbreviations: RVR, rapid virologic response; EVR, early virologic response; ETVR, end-of-treatment virologic response; SVR, sustained virologic response; HCV, Hepatitis C virus.

SDC5. Baseline plasma values of cytokines according to rs12980275 *IL28B* genotypes and HCV genotype (HCV GT) in 57 HIV/HCV coinfected patients.

	GG/AG		**AA**		***IL28B*/HCV GT**
	HCV GT1/4	**HCV GT3**	**HCV GT1/4**	**HCV GT3**	**p-value [(a)]**
Cytokine	**21**	**6**	**14**	**15**	
IFN-γ	7.34 (4.47 - 9.67)	7.35 (3.34 - 9.72)	4.69 (3.05 - 7.08)	4.07 (1.85 - 6.41)	**0.006**
IL-5	17.48 (11.57 - 22.37)	21.07 (10.86 - 32.07)	9.57 (6.41 - 16.36)	13.94 (10.82 - 19.46)	0.989
IL-9	1.73 (1.31 - 2.46)	1.67 (0.7 - 9.82)	1.39 (0.88 - 2.09)	1.2 (0.56 - 1.62)	**0.048**
TNF-α	10.04 (6.67 - 11.59)	4.56 (2.05 - 7.1)	5.48 (4.15 - 13.18)	5.19 (2.49 - 10.16)	0.228
IL-6	14.88 (2.42 - 17.03)	5.76 (2.07 - 8.28)	4.52 (1.84 - 17.03)	3.68 (2.24 - 13.73)	0.599

Values are expressed as median (P25th - P75th).
Statistically significant differences are shown in bold.
Only the most relevant and significant comparisons are shown.
(a), differences among patients from opposite groups: GG/AG, HCV GT 1/4 and AA, HCV GT3.
Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

SDC 6. Baseline plasma values of cytokines according to rs12980275 IL28B genotypes and sustained virologic response (SVR) in 57 HIV/HCV coinfected patients.

	GG/AG			AA		*IL28B*/SVR	
	SVR (-)	SVR (+)	p-value [(a)]	SVR (-)	SVR (+)	p-value [(b)]	p-value [(c)]
Cytokine	**17**	**11**		**8**	**21**		
IFN-γ	7.22 (4.1 - 9.1)	7.87 (5.71 - 10.34)	0.991	4.59 (2.7 - 7.35)	4.48 (2.57 - 6.37)	**0.016**	**0.038**
IL-5	17.01 (11.57 - 25.36)	19.46 (11.5 - 24.24)	0.994	10.72 (6.91 - 15.58)	13.68 (9.05 - 18.41)	0.369	0.862
IL-9	1.98 (1.48 - 2.46)	1.41 (0.69 - 2.06)	0.306	1.8 (1.17 - 2.17)	1.2 (0.62 - 1.57)	0.989	**0.001**
TNF-α	10.83 (6.91 - 12.43)	6.79 (2.1 - 10.04)	0.142	11.53 (5.45 - 14.59)	4.97 (2.89 - 8.9)	1.000	**0.023**
IL-6	15.82 (9.61 - 18.02)	2.24 (1.54 - 7.92)	**0.005**	9.36 (3.78 - 17.52)	2.78 (1.89 - 8.55)	0.995	**0.015**

Values are expressed as median (P25th - P75th).
Statistically significant differences are shown in bold.
Only the most relevant and significant comparisons are shown.
(a), differences in patients with GG or AG genotypes at rs12980275 *IL28B* according to SVR.
(b), differences in SVR(+) patients according to rs12980275 *IL28B* genotypes.
(c), differences among patients from opposite groups: GG/AG, SVR(-) vs. AA, SVR(+).
Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.