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ABSTRACT

Background: Toll-like receptor-3 (TLR3) is a cellular receptor that may recognize double-stranded RNA (dsRNA) from viruses, resulting in production of proinflammatory cytokines and interferons, which are important for the adaptive immune response.

Objectives: To analyze the association between *Toll-like receptor-3 (TLR3)* polymorphisms (rs3775291 and rs13126816) and virologic response to pegylated interferon-alpha plus ribavirin (pegIFNα/RBV) therapy in HIV/HCV coinfected patients.

Study design: We performed a retrospective study in 321 naïve patients treated with pegIFNα/RBV. Genotyping was performed by using the GoldenGate® assay with VeraCode®. The outcome variables were early virologic response (EVR) and sustained virologic response (SVR).

Results: In a multivariate analysis, rs3775291 A allele decreased the likelihood of achieving EVR (aOR=0.20; p=0.018) and SVR (aOR=0.38; p=0.024). Regarding rs13126816, the percentage of EVR decreased with each minor A allele (p=0.034) in HCV-GT2/3 patients, although no significant association was obtained in the multivariate analysis (p=0.076). Regarding *TLR3* haplotypes (comprised of rs3775291 and rs13126816), GT2/3 patients with AA haplotype had decreased odds of achieving EVR (p=0.030), whereas GG haplotype increased the likelihood (p=0.018). Regarding SVR, GG haplotype carriers had increased odds of achieving SVR (p= 0.019, p=0.043 and p=0.070 for all, GT2/3 and GT1/4 patients, respectively). Besides, GT1/4 patients with GA haplotype had lower odds of achieving SVR (p=0.039).

Conclusions: Our study shows the first evidence that two *TLR3* polymorphisms (rs3775291 and rs13126816) seem to be related to the HCV therapy response in HCV/HIV coinfected patients.

Key words: *TLR3;* polymorphisms; HIV; chronic hepatitis C; Interferon; HCV therapy

BACKGROUND

Hepatitis C virus (HCV) infection is an important cause of chronic liver disease and remains a major public health problem worldwide. The hepatitis C virus (HCV) therapy with pegylated-interferon-alpha plus ribavirin (pegIFNα/RBV) is still in use in HIV/HCV coinfected patients [1], even in combination with new direct-acting antivirals (DAAs) [2]. In fact, treatment with pegIFNα/RBV remains the only option of therapy for many patients in the world. The new DAAs are extremely expensive and there are serious restrictions for its administration, and in many regions in the world these drugs are inaccessible. Regarding HCV/HIV coinfected patients, although there is enough evidence to allow to these patients are treated, DAAs are still restricted. Moreover, it should be taken into account that the rate of HCV clearance after pegIFNα/RBV therapy in HIV/HCV coinfected patients is lower than in HCV monoinfected patients [3] and thus, the management of HCV infection supposes a significant challenge among HIV/HCV coinfected patients [4]. In the last years, several factors that influence the efficacy of IFN therapies have been identified, including age, sex, liver fibrosis, HCV genotype, HCV viral load, and obesity [5]. Additionally, the data reported in the scientific literature has showed that the response to IFN-based therapy is also related to *interleukin 28B* (*IL28B*) polymorphisms, particularly in patients infected with HCV genotype 1/4 [6]. However, an unexplained variability in pegIFNα/RBV treatment response still remains, which suggests that other host genetic factors may play an important role in pegIFNα/RBV therapy [7].

The toll-like receptors (TLRs) are a class of pattern recognition molecules with unique functions in the innate and the acquired immune systems [8]. The TLR3 is a cellular receptor that may recognize double-stranded RNA (dsRNA) from viruses in endosomal compartments or cell surfaces [9], stimulating a complex intracellular signalling cascade that results in production of proinflammatory cytokines and interferons, which are important for antiviral responses as well as induction of the adaptive arm of the immune system [9]. Moreover, *TLR* polymorphisms seem to play an important role in the immune response and pathogenesis of HCV infection [8]. In this context, *TLR3* rs3775291 polymorphism is a missense variant involved in impaired TLR3 signalling [10], which has been implicated in the outcome of numerous infections such as susceptibility to enteroviral myocarditis [11] and cytomegalovirus infection [12]; and protection against certain viral infections such as HIV-1 [13], herpes simplex virus type 2 (HSV-2) [14] and tick-borne encephalitis virus [15]. Regarding HCV infection, Askar et al. failed to find any association between rs3775291 and clinical parameters of chronic hepatitis C [16]; while Lee et al. and Citores et al. have recently described a relationship between rs3775291 and outcome in HCV patients after liver transplant [17, 18].

On the other hand, several reports have described an important role of the polymorphism rs13126816 on the susceptibility to certain viral infections and immune diseases such as herpes simplex virus type 2 infection [14] and type I diabetes mellitus [19]. Regarding HCV infection, in a recent article, Qian et al. found that rs13126816 had influence on the *TLR3* expression in macrophages, favouring the spontaneous HCV clearance in rs13126816 GG carriers [20].

OBJECTIVES

The aim of our study was to analyse the association between *TLR3* polymorphisms (rs3775291 and rs13126816) and virologic response to pegIFNα/RBV therapy in HIV/HCV coinfected patients.

STUDY DESIGN

Patients

We carried out a retrospective study on 321 HIV/HCV coinfected patients, who started HCV treatment with pegIFNα/RBV on regular follow-up from October 2000 to June 2010, at two reference HIV hospitals located in Madrid, Spain. The study was approved by the Research Ethic Committee of the Instituto de Salud Carlos III (ISCIII). This study was conducted in accordance with the Declaration of Helsinki and patients gave their written consent for the study.

The criteria for starting HCV antiviral treatment were: A) Inclusion criteria: chronic hepatitis C, HIV infection, 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/mm3, 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 [21, 22]. B) Exclusion criteria: Active opportunistic infections, active drug or alcohol addiction, and other concomitant diseases or conditions such as diabetes, nephropathies, autoimmune diseases, hemochromatosis, primary biliary cirrhosis, Wilson's disease, α1-antitrypsin deficiency, and neoplasia. Furthermore, we only included patients who fulfilled the HCV treatment and who had an available DNA sample for DNA genotyping.

Clinical data

Clinical and epidemiological data were obtained from medical records. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

Liver fibrosis was assessed by different methods, depending on the Hospital: a) At Hospital General Universitario "Gregorio Marañón" was used liver biopsy [23]; 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. b) At Hospital Carlos III was used transient elastometry (FibroScan®, Echosens, Paris, France) [24]; 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.

Following both international and national guidelines [3, 21, 22, 25], 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). Patients with HCV genotypes 1 or 4 (GT1/4) received either 48 or 72 weeks of treatment, and patients with HCV genotype 2 or 3 (GT2/3) were treated for 24 or 48 weeks, depending on the virologic response at week 4.

Laboratory data

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.

Genomic DNA was extracted from peripheral blood with Qiagen columns (QIAamp DNA Blood Midi/Maxi; Qiagen, Hilden, Germany). Two single nucleotide polymorphisms (SNPs) within the region of the *TLR3* gene (rs3775291 and rs13126816) were genotyped at the Spanish National Genotyping Center (CeGen; http://www.cegen.org/) by using GoldenGate® assay with VeraCode® Technology (Illumina Inc. San Diego, CA, USA).

Outcome variables

The virologic response to HCV treatment was measured by assessing serum HCV viral load at two different time points [3]: a) early virologic response (EVR): viral load dropped by 99% (2 $\log_{10}$) after 12 weeks of treatment; b) sustained virologic response (SVR): no detectable HCV viral load (<10 IU/mL) six months after treatment cessation.

Statistical analysis

The statistical analysis was performed by on-treatment approach. For the description of the study population, p-values were estimated with nonparametric tests: Mann-Whitney U test was used for continuous variable and Chi-square test for categorical variables. All SNPs were analysed for Hardy-Weinberg Equilibrium (HWE) by Chi-square test, considering equilibrium when $p>0.05$. For association analysis, logistic regression analysis was used to investigate the relationship among *TLR3* polymorphisms and HCV-therapy response. The model was adjusted by the most significant covariables: age, gender, baseline HCV-RNA viral load (<500,000 IU/mL vs. ≥500,000 IU/mL), significant fibrosis (F<2 vs. F≥2), *IL28B* rs12980275 polymorphism (AA vs. AG/GG), and HCV genotype (GT1/4 vs. GT2/3).The statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) 19.0 (SPSS INC, Chicago, IL, USA) (**Supplemental Table 1**).

In addition, pair-wise linkage disequilibrium (LD) analysis was computed by Haploview 4.2 software and haplotype-based association testing was performed using PLINK software (**Supplemental Table 1**). All p-values were two-tailed and statistical significance was defined as $p<0.05$.

RESULTS

Clinical characteristics

Table 1 shows the baseline characteristics of 321 HIV/HCV coinfected patients on HCV treatment. The median age was 42 years, 77.3% were males and 84.1% were on cART. The median baseline CD4+ count was 465 cells/mm^3, 66.8% had an HIV-RNA<50 copies/mL and 26.3% had an HCV-RNA<500,000 UI/mL. Regarding *IL28B* polymorphism (rs12980275), 46.1% had favorable AA genotype.

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

Characteristic	Values
No.	321
Male *	248 (77.3%)
Age (years) †	42 (38.6 – 45.9)
IVDU * **(**n=315)	282 (89.5%)
HAART *	270 (84.1%)
Anthropometric values	
Height (m) † (n=309)	1.70 (1.65 – 1.75)
Weight (Kgr) † (n=317)	67 (60 – 75.5)
BMI (kg/m^2) † (n=309)	23.1 (21.2 – 25.4)
BMI ≥25 kg/m ^{2} (n=309)	90 (29.1%)
HIV markers	
Nadir CD4+ T-cells/µL †	226 (131 – 335.5)
Nadir CD4+ <200 cells/µL *	137 (42.7%)
Baseline CD4+ T-cells/µL † (n=318)	465 (364.0 – 667.5)
Baseline CD4+ <500 T-cells/µL (n=318) *	175 (55.0%)
HIV-RNA <50 copies/ml (n=316) *	211 (66.8%)
HCV markers *	
HCV-genotype (n=320)	
1	182 (56.9%)
2	1 (0.3%)
3	100 (31.3%)
4	37 (11.6%)
HCV-RNA <500,000 IU/mL (n=315)	83 (26.3%)
Log$_{10}$ HCV-RNA (IU/mL) † (n=315)	6.09 (5.62 – 6.76)
***IL28B* polymorphism (rs12980275)**	.4)
AA	148 (46.1%)
AG	146 (45.5%)
GG	27 (8.4%)
Liver fibrosis (n= 289)*	
Significant fibrosis (F≥2)	182 (63.0%)
Advanced fibrosis (F≥3)	98 (33.9%)
Cirrhosis (F4)	51 (17.6%)

Values are expressed as: (*), absolute number (percentage); (†), median (interquartile range).

Abbreviations: BMI, body mass index; IVDU, intravenous drug users; HAART, highly active antiretroviral therapy; HCV, Hepatitis C virus; HCV-RNA, HCV serum viral load; HIV-1, Human immunodeficiency virus type 1; HIV-RNA, HIV plasma viral load.

TLR3 polymorphisms and association with virologic response

Allelic and genotypic frequencies of *TLR3* polymorphisms in our HIV/HCV coinfected patients are shown in **Supplemental Table 2**. The frequencies of rs3775291 and rs13126816 were in accordance with data listed on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs3775291 and http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs13126816, respectively). Both rs3775291 and rs13126816 polymorphisms were in HWE (p=0.192 and p=0.879, respectively), fulfilled the minimum allele frequency (MAF)>0.05, and displayed less than 5% of missing values. The LD was examined, and a weak LD was found between rs3775291 and rs13126816 (D'=0.72; r^2=0.36).

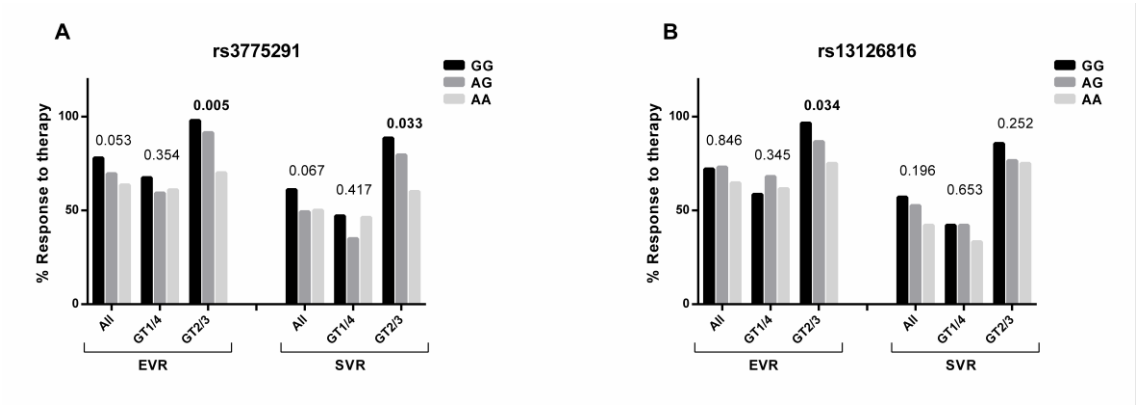

Figure 1. Frequencies of EVR and SVR according to TLR3 genotypes and HCV genotypes. P-values were calculated by linear-by-linear association Chi-squared test. **Abbreviations**: EVR, early virological response; SVR, sustained virological response; GT, HCV genotype.

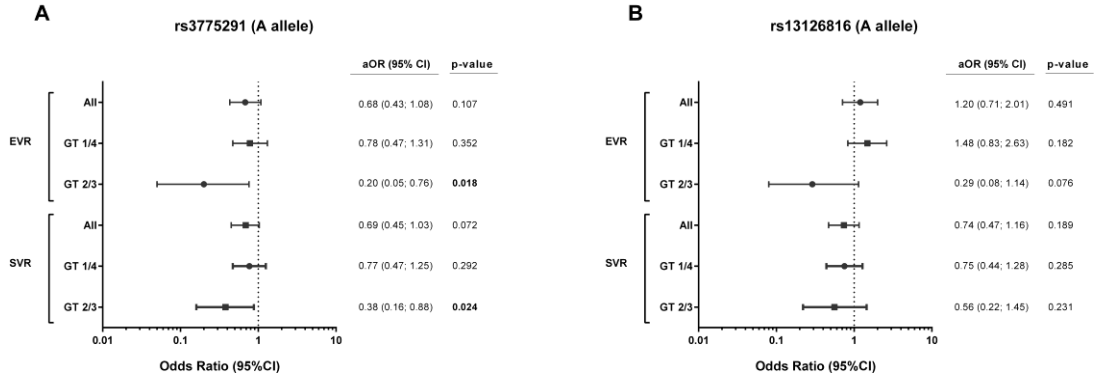

Figure 2. Association of *TLR3* polymorphisms (rs3775291 and rs13126816) with EVR and SVR in HIV/HCV coinfected patients on HCV therapy. The effect size is shown per A allele: the risk conferred by A allele is increased r-fold for heterozygotes (one copy of A allele) and 2r-fold for homozygotes (two copies of A allele). P-values were calculated by logistic regression analysis adjusted by the most important clinical and epidemiological characteristics (see statistical analysis in Patients and Methods section). Abbreviations: aOR, adjusted odds ratio; 95% CI, 95% confidence interval; EVR, early virological response; SVR, sustained virological response; GT, HCV genotype.

Figure 1 and **Figure 2** show the relationship between *TLR3* polymorphisms and the response to HCV treatment under an additive model of inheritance, which was the genetic model that best fitted our data. For rs3775291 (**Figure 1A**), the percentage of EVR and SVR in GT2/3 patients decreased with each minor A allele (p=0.005 and p=0.033, respectively). Furthermore, in the multivariate analysis, rs3775291 A allele decreased the likelihood of achieving EVR (adjusted odds ratio (aOR)=0.20; p=0.018) and SVR (aOR=0.38; p=0.024) in GT2/3 patients (**Figure 2A**). Regarding rs13126816, the percentage of EVR decreased with each minor A allele in GT2/3 patients (p=0.034), although statistical significance was lost in the multivariate analysis (aOR=0.29; p=0.076) (**Figure 2B**).

Association between TLR3 haplotypes and virologic response

Finally, we examined whether *TLR3* haplotypes (comprised of rs3775291 and rs13126816) were associated with HCV-treatment response (**Figure 3**).

For EVR (**Figure 3A**), GT2/3 patients with all unfavorable alleles (AA haplotype) had decreased odds of achieving EVR (aOR=0.21; p=0.030), whereas patients carrying all favorable alleles (GG haplotype) had increased odd of achieving EVR (aOR=4.51; p=0.018).

Regarding SVR (**Figure 3B**), GG haplotype carriers had increased odds of achieving SVR when taking into account all patients (aOR=1.64; p=0.019) and GT2/3 patients (aOR=2.32; p=0.043), as well as a trend toward significance in GT1/4 patients (aOR=1.58; p=0.070). Besides, GT1/4 patients with GA haplotype had lower odds of achieving SVR (aOR=0.29; p=0.039).

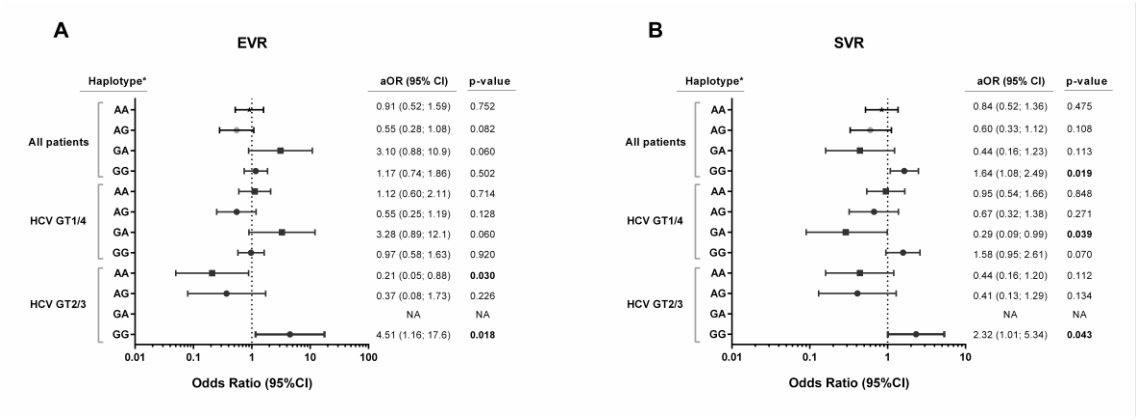

Figure 3. Association of *TLR3* haplotypes with EVR and SVR in HIV/HCV coinfected patients on HCV therapy. P-values were calculated by logistic regression analysis adjusted by the most important clinical and epidemiological characteristics (see statistical analysis in Patients and Methods section). Abbreviations: aOR, adjusted odds ratio; 95% CI, 95% confidence interval; EVR, early virological response; SVR, sustained virological response; GT, HCV genotype; NA, not available due to a low number of patients in one of the groups. *Haplotypes are composed of one rs3775291 allele plus one rs13126816 allele.

DISCUSSION

In this study, we found the minor A allele at both *TLR3* polymorphisms was related to decreased odds of achieving a virologic response to HCV therapy, whereas the major G allele was related to significant higher proportion of successful virologic response.

TLR3 polymorphisms have been related to numerous infectious diseases, showing a key role in the innate immune response and the pathogenesis of these diseases. In our study, the odds of achieving both EVR and SVR decreased for each minor A allele at rs3775291. The rs3775291 polymorphism (within exon 4) leads to a missense mutation, changing leucine to phenylalanine at amino acid position 412 (G/A, Leu412Phe) in the *TLR3* gene [26]. This polymorphism seems not to affect the level of *TLR3* expression or its intracellular location in vesicles, but seems to have a negative effect on the binding capacity to dsRNA [27], and the cell surface expression of TLR3, although discrepant results have been described [10, 27]. However, minor A allele has been associated with reduced NF-kappaB activation to half of the G allele level [10] as well as reduced type I interferon signalling [11], which could also lead to decreased production of cytokines and chemokines. These previous findings could partly explain our results. In this setting, the presence of the minor A allele might contribute to an impaired activation of immune response with subsequent reduced production of antiviral molecules such as type I interferon, leading to a lower response to IFN-based therapy. Concurrently, patients carrying the major G allele could have a higher capacity of response to clarify the HCV infection, as shown by our results.

The rs13126816 polymorphism is located within intron 1 of the *TLR3* gene. In our study, carriers of rs13126816 G allele infected with GT2/3 had higher proportion of EVR. However, as we failed to find a significant association after adjusting by the most significant covariates, further studies with larger population size would be interesting to confirm the true role of this SNP according to treatment-related HCV clearance.

Moreover, in our study, *TLR3* haplotypes (formed by rs3775291 and rs13126816 polymorphisms) were also investigated to analyze the association with EVR or SVR. The GG haplotype (favorable alleles) was associated with SVR when considered all patients and in GT2/3 patients, and was close to statistical significance in GT1/4 patients. These associations confirm the idea that *TLR3* polymorphisms seem to have an influence on virologic response to HCV treatment. Moreover, while the association between individual *TLR3* polymorphisms and virologic response was not found for GT1/4 patients, *TLR3* haplotypes showed a trend towards significance, which suggest that *TLR3* polymorphisms could also have impact on SVR among patients infected with GT1/4. However, additional studies with higher size-population would be necessary to corroborate the role of these polymorphisms among GT1/4 patients.

The *IL28B* polymorphisms have been widely related to virologic response to HCV therapy [6]. It should be noted that TLR3 is also involved in the activation of interferon regulatory factor (IRF), which is a crucial transcriptional factor for the induction of *IL28A* and *IL28B* genes [28]. Thus, it could be possible that *TLR3* polymorphisms could indirectly have an effect on the well-known relationship

between *IL28B* polymorphisms and SVR [6, 29]. However, it should be noted that logistic regression analysis was performed adjusting for an *IL28B* polymorphism (rs12980275), finding *TLR3* polymorphisms were associated with response to HCV treatment independently of *IL28B* polymorphisms.

Finally, for the correct interpretation of the data, it must be taken into account that the study design was retrospective and the number of patients was limited, which could limit the achievement of statistically significant values. Thus, it would be interesting to perform further studies with larger population for corroborating our findings. Secondly, note that most of the patients in GT2/3 group were GT3, thus results in this group are mainly applicable to GT3 patients. Thirdly, HCV therapy regimens were not identical for all patients. Fourthly, this study was performed on patients with European ancestry, and it would be interesting to perform these analyses on different ethnic groups. Finally, our study included only HIV/HCV coinfected patients, and it would also be interesting to know the role of studied *TLR3* SNPs in HCV monoinfected patients.

In conclusion, our study shows the first evidence that two *TLR3* polymorphisms (rs3775291 and rs13126816) seem to be related to the HCV therapy response in HCV/HIV coinfected patients, mainly in GT2/3 infected patients. However, further analyses are needed to determine the potential use of *TLR3* polymorphisms as a predictive marker of HCV therapy response.

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COMPETING INTERESTS:

The authors do not have a commercial or other association that might pose a conflict of interest.

ETHICAL APPROVAL:

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

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SUPPLEMENTARY DATA

Supplemental Table 1. Computational steps of statistical analyses

Scripts for Figure 1 with SPSS v21 software

```
CROSSTABS
  /TABLES= EVR
  BY rs13126816_nA rs3775291_nA
  BY HCV-GT
  /FORMAT=AVALUE TABLES
  /STATISTICS=CHISQ
  /CELLS=COUNT COLUMN
  /COUNT ROUND CELL.
CROSSTABS
  /TABLES= SVR
  BY rs13126816_nA rs3775291_nA
  BY HCV-GT
  /FORMAT=AVALUE TABLES
  /STATISTICS=CHISQ
  /CELLS=COUNT COLUMN
  /COUNT ROUND CELL.
```

Scripts for Figure 2 with SPSS v21 software

```
LOGISTIC REGRESSION VARIABLES EVR
   /METHOD=ENTER rs3775291_nA Age Gender HCV500000 F2 AA_rs12980275 HCV-GT14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
LOGISTIC REGRESSION VARIABLES SVR
   /METHOD=ENTER rs3775291_nA Age Gender HCV500000 F2 AA_rs12980275 HCV-GT14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
LOGISTIC REGRESSION VARIABLES EVR
  /METHOD=ENTER rs13126816_nA Age Gender HCV500000 F2 AA_rs12980275 GrupoG14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
LOGISTIC REGRESSION VARIABLES SVR
  /METHOD=ENTER rs13126816_nA Age Gender HCV500000 F2 AA_rs12980275 GrupoG14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
SPLIT FILE OFF.

SORT CASES BY GrupoG14.
SPLIT FILE LAYERED BY GrupoG14.
LOGISTIC REGRESSION VARIABLES EVR
   /METHOD=ENTER rs3775291_nA Age Gender HCV500000 F2 AA_rs12980275 HCV-GT14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
LOGISTIC REGRESSION VARIABLES SVR
   /METHOD=ENTER rs3775291_nA Age Gender HCV500000 F2 AA_rs12980275 HCV-GT14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
LOGISTIC REGRESSION VARIABLES EVR
  /METHOD=ENTER rs13126816_nA Age Gender HCV500000 F2 AA_rs12980275 GrupoG14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
LOGISTIC REGRESSION VARIABLES SVR
  /METHOD=ENTER rs13126816_nA Age Gender HCV500000 F2 AA_rs12980275 GrupoG14
  /CRITERIA=PIN(.05) POUT(.10) ITERATE(20) CUT(.5).
SPLIT FILE OFF.
```

SORT CASES BY Nº_DNA(A).

Scripts for Figure 3 with PLINK software

plink --file ./TLR/X1 --make-bed --out TLR/X1 --noweb

plink --bfile ./TLR/X1 --hap-snps rs13126816-rs3775291 --chap --ci 0.95 --specific-haplotype AA --covar ./TLR/covar.txt --out TLR/X1_AA --noweb
plink --bfile ./TLR/X1 --hap-snps rs13126816-rs3775291 --chap --ci 0.95 --specific-haplotype GA --covar ./TLR/covar.txt --out TLR/X1_GA –noweb
plink --bfile ./TLR/X1 --hap-snps rs13126816-rs3775291 --chap --ci 0.95 --specific-haplotype AG --covar ./TLR/covar.txt --out TLR/X1_AG –noweb
plink --bfile ./TLR/X1 --hap-snps rs13126816-rs3775291 --chap --ci 0.95 --specific-haplotype GG --covar ./TLR/covar.txt --out TLR/X1_GG –noweb

being X1= SNPs file; covar= covariate file; X1_AA,X1_GA, X1_AG,X1_GG= output files