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Relating the outcome of HCV infection and different host SNP polymorphisms in a Majorcan population coinfected with HCV–HIV and treated with pegIFN-RBV

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Summary. Hepatitis C virus (HCV) is one of the major causes of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, and the development of HCV-related disease is accelerated in individuals coinfected with human immunodeficiency-1 virus (HIV). In the present study, we correlated different host single-nucleotide polymorphisms (SNPs) in the *IL28B*, *CTLA4*, *LDLr*, and *HFE* genes and mitochondrial DNA (mtDNA) haplogroups with the outcome of HCV infection and the response to pegylated-interferon plus ribavirin (pegIFN-RBV) treatment. Our study population consisted of 63 Majorcan patients coinfected with HCV and HIV and 59 anonymous unrelated controls. Whereas the population frequency of *IL28B* alleles was similar to that found in a North-American cohort of European descent, the frequency of the *rs12979860 C* allele was lower than that determined in other cohorts from Spain. The frequencies of *CTLA4* and *LDLr* polymorphisms were comparable to those reported in other populations. Significant differences between cases and control cohorts occurred only for the H63D mutation of the *HFE* gene. There were no other differences in the frequencies of other polymorphisms or mtDNA haplogroups. The *IL28B rs12979860* CC genotype was shown to be associated with a rapid virological response, and the spontaneous viral clearance rate for HCV was higher in patients with the *CTLA4+49* G allele. There was no relationship between SNPs in the *LDLr* and *HFE* genes and mtDNA haplogroups and the response to treatment. Our results suggest that the host genetic background plays a significant role in the pegIFN-RBV response of patients coinfected with HCV and HIV. [**Int Microbiol** 2014; 17(1):11-20]

Keywords: HCV–HIV co-infection · mtDNA haplogroups · SNP polymorphisms

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Introduction

Hepatitis C virus (HCV) is the main cause of chronic liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma [2]. The World Health Organization (WHO) estimates that up to 3 % of the world's population is infected

with HCV, implying that there are 170–200 million carriers of the virus [18,33]. The prevalence of HCV infection ranges from less than 1 % in Northern Europe to more than 2 % on average in North Africa. The highest HCV prevalence (*ca*. 15 %) is in Egypt [21]. HCV transmission occurs primarily through transfusions of contaminated blood or blood products and in drug abusers through the sharing of contaminated needles and syringes.

Because of the common routes of transmission, HCV-infected patients are also frequently infected with the human immunodeficiency virus (HIV). HIV positivity has been recognized as an important factor responsible for an increased morbidity and mortality in HCV-infected individuals, and antiviral treatment for HIV modifies HCV disease outcome and survival [1,33]. Although spontaneous virus clearance (SVC) occurs in 15–30 % of HCV-infected adults, in 75–85 % of patients the infection progresses to chronic disease. Children and young women have a higher rate of SVC, around 45 % of the total number of infected individuals in these groups [32]. For many years, the most effective treatment for HCV infection has been based on the administration of interferon (IFN), which produces a sustained viral response (SVR) in 20 % of HCV-infected patients. The combination of IFN with ribavirin (RBV) and the subsequent addition of polyethylene glycosylated IFN (pegIFN) was a breakthrough in the treatment of HCV-mediated disease, increasing the response rate to 50–80 %. Patients who fail to show an early decline in viral load during treatment are less likely to achieve a SVR [3]. In addition, patient characteristics such as older age, male gender, overweight, and the presence of cirrhosis or hepatic steatosis, insulin resistance, diabetes, or coinfection with HIV or hepatitis B virus (HBV) [3,13,27,29] are all associated with a lower rate of SVR.

In HCV-infected patients, an important aspect of clinical practice is monitoring the efficacy of treatment and the duration of the effectiveness, through repeated measurements of HCV RNA levels. These measurements are typically performed before treatment is started, at 4, 12, and 24 weeks of treatment and, finally, 24 weeks after the end of the treatment, to determine whether the patients has achieved SVR. The probability of achieving a SVR is inversely proportional to the time until the viral load becomes undetectable. Treatment should be discontinued at week 12 if the decrease in viral load is less than 2 logs (IU/ml) and at week 24 if there is still detectable viral load, as in these patients the probability of a SVR is very low (1–3 %) [6].

Identification of the mechanisms underlying treatment failure in patients receiving pegIFN-RBV would be useful to anticipate the likelihood of their achieving a SVR. Different single nucleotide polymorphisms (SNPs) located upstream of the interleukin 28B gene (*IL28B*) have been related to the outcome of HCV infection and to the success of peg-IFN-RBV therapy. For example, Ge et al. [9] have found a strong relation between the SNP *rs12979860*, located close to the *IL28B* gene, and treatment response.

A large number of SNPs in other genes have also been studied for their association with disease outcome, including: (i) immune-related genes, such as the cytotoxic T-lymphocyte antigen 4 gene (*CTLA4*) [5,24,31,36]; (ii) lipid metabolism genes, such as the low density lipoprotein receptor gene (*LDLr*) [19,22,30,35]; and (iii) SNPs implicated in other liver diseases, such as mutations in the hereditary hemochromatosis gene (*HFE*) [4,10,16]. Other studies have used population-related markers such as mtDNA haplogroups, which are also an indicator of mitochondrial function [8,14,20].

In this study, we examined the frequencies of the SNPs associated with treatment response in a cohort of HCV–HIV-coinfected patients in Majorca. Specifically, we analyzed the frequencies of SNPs in the *IL28B*, *CTLA4*, *LDLr*, and *HFE* genes and the mtDNA haplogroups and whether either one correlated with the outcome of HCV infection in patients treated with pegIFN-RBV. At the same time, we determined whether the frequencies of these SNPs in our Majorcan cohort were similar to those in other populations.

Materials and methods

Patient and control samples. Sixty-three HCV–HIV coinfected patients were selected from those attending the Infectious Diseases Unit of Son Llàtzer Hospital, Palma, Balearic Islands, Spain. Inclusion criteria were positive viremia for both viruses (HCV and HIV) and seronegativity (HBsAg negative) for HBV. Six patients showed spontaneous HCV clearance while being evaluated for inclusion for HCV treatment. Spontaneous HCV clearance was defined as HCV-RNA negativity but HCV seropositivity in a treatment-naive patient. Fifty-seven patients were treated for 48 weeks with pegIFN-RBV: pegIFNα-2a: 180 μg/week or peg-IFNα-2b: 1.5 μg/kg/week and ribavirin according to patient weight. Patients weighing < 75 kg received 1000 mg/day (2–0–3), and those weighing >75 kg, 1200 mg/day (3–0–3).

Buccal epithelial cells were obtained from three oral swabs and then air-dried and stored at room temperature. DNA samples from 59 anonymous control individuals (29 males and 30 females) from the general Majorcan population were randomly selected from those available in the Genetics Laboratory of the University of the Balearic Islands. The study was approved by the local institutional ethics committee and all patients provided informed consent regarding the use of their biological samples for medical research.

Variables. We constructed a database with patient variables collected retrospectively by chart review. The variables consisted of the basic characteristics of the patients (age, sex, body mass index, alcohol consumption). Other variables were biochemical markers, including aspartate transaminase (AST), alanine transaminase (ALT), and cholesterol, blood count (hemoglobin, leukocytes, platelets), features related to infection, such as HCV genotype, HCV

Table 1. Host genetic polymorphisms analyzed

Gene	Polymorphism	Nucleotide change
Interleukin 28B (*IL28B*)	*rs12979860*	C/T
Cytotoxic T-lymphocyte antigen 4 (*CTL4*)	+49 (*rs231775*)	G/A
	–318 (*rs5742909*)	C/T
Low density lipoprotein receptor (*LDLr*)	*rs14158*	A/G
Hereditary hemochromatosis gene (*HFE*)	C282Y	G/A
	H63D	C/G
	S65C	T/A
Mitochondrial DNA haplogroup	H	
	Others (non-H)	

baseline viral load, transmission route, treatment with highly active antiretroviral therapy (HAART), and variables related to effects of the disease. The latter included CD4+ T-cell count, liver damage measured by fibroscan (transient elastography) and biopsy, and the presence of hepatomegaly, splenomegaly, and/or hepatic steatosis. Fibrosis stage was obtained by biopsy (stage F0–F1) and/or fibroscan measurements (values <7.2 kPa were also considered as F0–F1 stage). Biopsy data were taken into account when both measurements (biopsy and fibroscan) were available. Response to treatment was evaluated at 4, 12, 24, and 48 weeks (SVR). Patients were classified as having achieved a SVR when the viral load remained undetectable 24 weeks after the completion of treatment (week 48).

Genetic polymorphisms. The SNPs *IL28B rs12979860*, *CTLA4*+49 *rs231775*, *CTLA4*–318 *rs5742909*, *LDLr rs14158*, *HFE* C282Y, *HFE* H63D, and *HFE* S65C were evaluated, together with the mtDNA haplogroup. In the control samples from the Majorcan population, only the polymorphisms not determined in previous studies were evaluated, i.e., *IL28B*, *CTLA4*+49, *CTLA4*-318, *LDLr*, and *HFE* S65C (Table 1). Genotyping was carried out using restriction fragment length polymorphism (RFLP) techniques and, when necessary, DNA sequencing (for *LDLr* SNPs and non-H mtDNA haplogroups).

DNA extraction and amplification. DNA was extracted from the oral swabs using a standard phenol-chloroform extraction method followed by precipitation with ethanol and NaCl. The primers designed and used in the present work to amplify the *IL28B* upstream region and the *LDLr* were, respectively, IL28B-forward 5′-GCTTATCGCATACGGCTAGG-3′ and IL28B-reverse 5′-AGGGACCGCTACGTAAGTCA-3′ (427 bp amplicon), and LDLr-forward 5′-TGGCAGAGACAGATGGTCAG-3′ and LDLr-reverse 5′-CACTGTCCGAAGCCTGTTCT-3′ (195 bp amplicon). The primers used to amplify *CTLA4* (680 bp) and *HFE* gene (390 bp for C282Y and 208 for H63D/S65C) polymorphisms were those described by Nischalke et al. [24] and Feder et al. [7], respectively. PCRs were carried out using 40 ng of DNA, 0.2 pM of each primer, 0.2 mM of each dNTP, and 0.75 units of Taq polymerase (Dynazyme, Thermo Fisher, Lafayette, CO, USA). Amplification consisted of denaturation at 94ºC for 10 min followed by 35 cycles of denaturation at 94 ºC for 1 min, 1 min at the annealing temperature (59–60 ºC), and 1 min of extension at 72 ºC, with a final extension at 72 ºC for 10 min.

The RFLP motif -7025 *Alu*I was analyzed to determine mtDNA haplogroup H using the following primers: 5′-CCGTAGGTGGCCTGACTGGC-3′ (forward) and 5′- TGATGGCAAATACAGCTCCT-3′ (reverse) [25]. PCR (124 bp) was carried out using 60 ng of genomic DNA, 2 pM of each primer, 0.4 mM of each dNTP, and 1.25 units of Taq polymerase (Dynazyme). This PCR conditions were denaturation at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 50 s, 1 min 50 s at 59 ºC (annealing), and 1 min of extension at 72 ºC; 5 min at 72 ºC was added at the end of the cycles.

The mtDNA hypervariable regions of non-H samples (a 453-nucleotide sequence in region I and a 401-nucleotide in region II) were analyzed using the primers L15996 (5′-CTCCACCATTAGCACCCAAAGC-3′) and H16401 (5′-TGATTTCACGGAGGATGGTG-3′) for hypervariable region I, and L48 (5′-CTCACGGGAGCTCTCCATGC-3′) and H408 (5′-CTGTTAAAAGTG-CATACCG CCA-3′) for hypervariable region II [34]. Each PCR consisted of 80 ng of DNA, 0.2 pM of each primer, 0.4 mM of each dNTP, and 1 unit of Taq polymerase (Dynazyme), with denaturation at 96 ºC for 5 min followed by 30 cycles of denaturation at 94 ºC for 1 min, 1 min at 50 ºC (annealing), and 1 min of extension at 72 ºC.

Genotyping by RFLPs. Table 2 shows the restriction enzymes, cutting sites, conditions used in the RFLP digestions, and the resulting band patterns. RFLP assays not described in the literature were designed with the tools provided on the New England Biolabs website [http://tools.neb.com/NEBcutter2/]. Digested DNA was electrophoresed in EEO agarose with 1× TAE buffer, using ethidium bromide for DNA staining. To verify the results of the new RFLP assays, direct sequencing of the target SNP was performed from PCR amplicons.

Genotyping by sequencing. Polymorphisms in the *LDLr* gene and the non-H mtDNA haplogroups were detected by DNA sequencing. The PCR products were purified using the PCRapace spin kit (Invitek, Berlin, Germany) and sequenced with the BigDye Terminator v.3.1. cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The samples were precipitated with ethanol and analyzed in an ABI 3130 automated sequencer (Applied Biosystems). The chromatograms and outputs were analyzed using the Bioedit Sequence Alignment Editor software [12]. Non-H mtDNA sequences were aligned and the most probable haplogroup was assigned by means of the Haplogrep software [haplogrep.uibk.ac.at].

Statistical methodology. The genotypic and allelic frequencies of the polymorphisms were calculated by direct counting. Chi-square or Fisher's tests, when appropriate, were performed on contingency tables to identify differences in genotype distributions between cases and controls. To analyze associations with qualitative variables (sex, genotype virus, route of transmission, biopsy data, viral load, hepatomegaly, splenomegaly, and hepatic steatosis), the chi-square statistic or Fisher's test was also used on contingency tables. The association of the host genotypes with a response to treat-

Table 2. Conditions and band patterns in RFLP analyses

Table 2A. Conditions

Polymorphisms	Enzymes	Target point (polymorphic nucleotide)	Temperature and time	Agarose
IL28B rs12979860	*Hpy*166II	G**T**N^NAC	37 °C – 3 h	2 %
CTLA4 +49	*Fnu*4HI	GC^N**G**C	37 °C – 2 h	4 %
CTLA4 –318	*Mnl*I	CCT**C**$(N)_7$^	37 °C – 2 h	4 %
HFE C282Y	*Rsa*I [1]	GT^**A**C	37 °C – 3.5 h	3 %
HFE H63D	*Bcl*I [1]	T^GAT**C**A	50 °C – 2h	3 %
HFE S65C	*Hinf*I	G^AN**T**C	37 °C – 3h	3 %
Haplogrup H	*Alu*I	AG^C**T**	37 °C – 3h	

Table 2B. Band patterns

Polymorphisms	Motif	bp fragment	Motif	bp fragment	Unspecific fragment (bp)
IL28B rs12979860	C	320	T	28, 292	
CTLA4 +49	A	223	G	28, 195	457
CTLA4 –318	C	59, 71	T	130	53, 111, 376
HFE C282Y	Normal	250	Mutated	110, 140	
HFE H63D	Normal	70, 138	Mutated	208	
HFE S65C	Normal	58, 150	Mutated	208	
Haplogrup H	Haplo H	124	Non-H	80	

[1]Ref. Lynas [17].

ment (at 4, 12, and 24 weeks, SVR) or with spontaneous clarification was tested following the same approach.

Quantitative variables were tested for a normal distribution by the Kolmogorov-Smirnov test. Variables with a normal distribution were analyzed using Student's *t* test or an ANOVA when comparing two or three groups, respectively. Quantitative variables that did not follow a normal distribution were analyzed using the nonparametric Mann-Whitney or Kruskal-Wallis test, for comparisons of two or three groups, respectively. All statistical calculations were performed using SPSS software for Windows (Rel. 15.0.1 2006 Chicago: SPSS Inc.).

Results

Baseline characteristics of the subjects. The main characteristics of the study subjects are shown in Table 3. Variables with a normal distribution were age, body mass index, cholesterol, hemoglobin levels, platelets, leukocytes and fibrosis assessed by fibroscan; quantitative variables that did not follow a normal distribution were AST, ALT, HCV viral load, and the absolute CD4 count. Gender was not evenly distributed as there were twice as many male as female patients. Males had higher levels of AST ($P < 0.05$), ALT ($P < 0.05$), and hemoglobin ($P < 0.005$) than females. Current injection drug usage was the main route of transmission, but it did not correlate with the degree of fibrosis. Relevant alcoholic consumption (> 50 mg/day for over 2 years) was present only in a few female patients. In the treated patients, AST and ALT levels and the frequency of HCV genotype 3 infection (all with a $P < 0.05$) were higher in patients with a SVR. Six out of 63 patients (9.5 %) had SVC during treatment evaluation and were therefore not treated with pegIFN-RIB. AST and ALT levels were significantly lower in patients with SVC than in treated patients ($P < 0.005$).

Frequencies of the genetic polymorphisms. All of the studied host genetic polymorphisms were unequivocally assigned in all cases. *IL28B*, *LDLr*, *CTLA4*, and *HFE* (S65C) polymorphisms were analyzed in 53–58 individuals of the control group, depending on the SNP. The distribution

Table 3. Baseline demographic, epidemiological and clinical characteristics of HCV–HIV coinfected subjects. Frequencies and proportions or means plus standard deviations (in parenthesis) are indicated in categorical and continuous variables, respectively.

		Treated patients (n = 57)				SVC patients (n = 6)	All patients (n = 63)	
		SVR (n = 24)	no SVR (n = 31)	Total				
AGE, years		42.42 (5.55)	40.77 (4.92)	41.53 (5.43)		41.67 (6.31)	42 (5)	
BMI (na = 4)		24.74 (3.01)	23.58 (3.12)	24.10 (3.10)		22.64 (3.00)	23.97 (3.09)	
Cholesterol, mg/dl (na = 2)		175.70 (41.05)	174.26 (34.67)	174.57 (36.54)		212.40 (64.96)	178 (40)	
AST level, U/ml (na = 1)		76.29 (45.15)	58.52 (44.04)	66.02 (44.65)	$P < 0.05$	24.4 (8.20)	63 (44)	$P < 0.005$
ALT level, U/ml (na = 1)		95.17 (57.79)	70.48 (56.76)	81.40 (57.56)	$P < 0.05$	27.6 (11.31)	77 (57)	$P < 0.005$
CD4 cell count, cell/mm^3 (na = 1)		468.33 (234.21)	582.55 (339.92)	526.95 (298.35)		375.40 (148.94)	515 (291)	
Haemoglobin levels, g/dl (na = 11)		14.81 (1.70)	14.26 (1.48)	14.55 (1.58)		13.42 (1.66)	14.44 (1.61)	
Platelet count, 10^9/L (na = 1)		182.71 (57.37)	208.48 (63.37)	197.18 (60.84)		242.00 (30.16)	200.79 (6.08)	
Leukocytes, 10^3/mm^3 (na = 12)		5.90 (2.16)	5.87 (1.60)	5.84 (1.81)		5.60 (1.14)	5.81 (1.75)	
Gender (na = 2)	Male	20	19	39		2	41 (67.2)	
	Female	4	12	16		4	20 (32.8)	
HCV viral load (na = 8)	Low	8	12	20		–	20 (36.3)	
	High	16	19	35		–	35 (63.7)	
HCV genotypes (na = 9)	1	9	15	24		–	24 (44.4)	
	3	12	5	17	$P < 0.05$	–	17 (31.5)	
	4	2	11	13		–	13 (24.1)	
Fibrosis stage (Metavir) (na = 12)	F0-F1	3	10	13		2	15 (29.4)	
	F2-F4	18	18	36		0	36 (70.6)	
Alcohol consump. (na = 6)	Yes	2	1	3		0	3 (5.2)	
	No	16	25	41		5	46 (80.7)	
	Abstinent	5	3	8		0	8 (14.0)	
Route of Transmission (na = 5)	IDU	21	22	43		5	48 (82.8)	
	Other	2	7	9		1	10 (17.2)	
Highly active antiretroviral therapy (na = 2)	Yes	21	29	50		4	54 (91.5)	
	No	2	2	4		1	5 (8.5)	
Hepatomegaly (na = 8)	Yes	3	6	9		2	11(20.0)	
	No	20	23	43		1	44 (80.0)	
Hepatic steatosis (na = 20)	Yes	4	5	9		1	10 (23.3)	
	No	14	18	32		1	33 (76.7)	
Splenomegaly (na = 8)	Yes	5	8	13		1	14 (25.5)	
	No	18	21	39		2	41 (74.5)	

Note: BMI: body mass index. AST: aspartate transaminase. ALT: alanine transaminase. Alcohol consumption: Yes: >50g for >2 years; Abstinent: no alcohol consumption for 6 month. HCV viral load: High: >600,000 IU/ml: Routes of transmissions: heterosexual or homosexual contact and transfusions. Fibrosis stage: data from biopsy and Fibroscan (F0-F1 and <7.2 kpa).na: not available data. SVR: Sustained viral response. SVC: Spontaneous viral clearance.

Table 4. Polymorphism distribution in patients (treated *versus* SVC) and control cohorts

		Patients			
Polymorphisms		Treated	SVC	Total	Controls (%)
IL28B rs12979860	CC	19	4	23	19 (35.8)
	CT	29	2	31	27 (50.9)
	TT	9	0	9	7 (13.2)
CTLA4 +49	AA	29	0	29	30 (54.6)
	AG	23	5	28	18 (32.7)
	GG	5	1	6	7 (12.7)
CTLA4 -318	CC	49	6	55	51 (87.9)
	CT	7	0	7	6 (10.4)
	TT	1	0	1	1 (1.7)
HFE C282Y	N	53	6	59	181 (94.3)*
	Hz	4	0	4	11 (5.7)*
HFE H63D	N	21	4	25	120 (62.5)*
	Hz	32	2	34	69 (35.9)*
	Ho	4	0	4	3 (1.6)*
HFE S65C	N	55	5	60	51 (96.2)
	Hz	2	1	3	2 (3.8)
LDLr	GG	30	3	33	28 (51.8)
	AG	21	3	24	19 (35.2)
	AA	6	0	6	7 (13)
mtDNA haplogroups	H	25	3	28	14 (32.6)**
	J	9	1	10	4 (9.3)**
	U	6	2	8	6 (14.0)**
	Other	17	0	17	19 (44.2)**

*Data from Guix et al. [11]. **Data from Picornell et al. [25]. N = normals; Ho = homozygotes; Hz = heterozygotes.

of the polymorphisms is shown in Table 4. All of the polymorphisms were in Hardy-Weinberg equilibrium and there was no correlation among the variations in the SNPs. The allele distribution between patients and controls differed only for the H63D mutation (*HFE* gene), with the heterozygote genotype occurring more frequently in the patient cohort than in the controls ($P = 0.002$). Mitochondrial haplogroups were grouped into four principal groups H, J, U, and the rest of the haplogroups.

Statistical analyses showed that patients with the *IL28B rs12979860 CC* genotype had lower levels of leukocytes ($P = 0.047$) and platelets ($P = 0.004$), although for the former the difference was less when an outlier patient was removed from the analysis. In addition, HCV genotype 4 infection was less frequent in patients with the *IL28B rs12979860 CC* genotype ($P = 0.049$). The relationships were most evident when heterozygous CT and homozygous TT genotypes were grouped and compared with the CC genotype. Moreover, ALT and AST transaminase levels were substantially higher in patient carriers of the G allele in *CTLA4*+49 ($P = 0.021$ and $P = 0.063$, respectively) than in patients with the AA genotype. The fibroscan values (fibrosis) of patients carrying the CC genotype of *CTLA4*–318 were above average, although there were no differences when fibrosis measurements by biopsy and fibroscan were pooled under the same variable. Spontaneous HCV clearance was higher in patients carrying the G allele in the *CTLA4*+49 polymorphism ($P = 0.02$) (Table 4 and Fig. 1B). For *LDLr*, patients with the AA genotype were more likely to have hepatic steatosis ($P = 0.047$) and a lower body mass index ($P = 0.029$) than those with the AG or GG genotypes. None

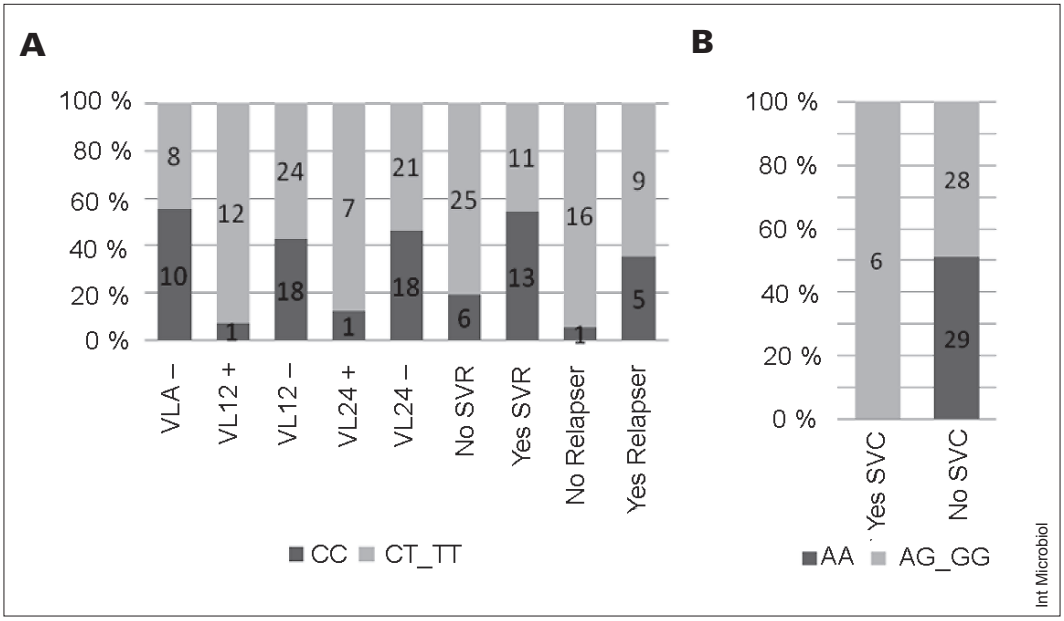

Fig. 1. **(A)** Grouped *IL28B* genotype (CC and CT_TT) and response to treatment. **(B)** Grouped *CTLA4+49* genotype (AA and AG_GG) and spontaneous viral clearance (SVC). The number of patients is indicated.

of the patients were homozygous for the C282Y mutation in the *HFE* gene, and the prevalence of the mutation S65C was extremely low in both controls and patients. Besides the different prevalences of the H63D mutation in patients vs. controls, the most remarkable feature was that three of the four C282Y/wt patients, the H63D/H63D patients, and the S65C/wt patients had previously undergone liver biopsy, which suggests a suspicion of liver involvement. Hepatomegaly was a common feature of patients belonging to the major haplogroups H, J, and U. Finally, ALT levels were significantly higher in patients with mtDNA haplogroup H and those levels were also higher, but not significantly, in carriers of haplogroup J.

Table 5. Distribution of the response to pegIFN-RBV treatment in males and females of the patient cohort

		Male	Female	Total
VR4	Negative	14	4	18 (40.9 %)
	Positive	19	7	26 (59.1 %)
VR12	Negative	30	8	38 (69.1 %)
	< 2 log	3	1	4 (7.3 %)
	Positive	8	5	13 (23.6 %)
VR24	Negative	31	8	39 (83.0 %)
	Positive	5	3	8 (17.0 %)
SVR	No	19	12	31 (56.4 %)
	Yes	20	4	24 (43.6 %)
SVC	No	41	16	57 (90.5 %)
	Yes	2	4	6 (9.5 %)

VR4, VR12, VR24: Viral load after 4, 12 and 24 weeks.
Negative: non detectable viral load; <2 log: viral load has decreased more than two log.
Positive: viral load has not significantly changed.
SVR: Sustained viral response 24 weeks after treatment.
SVC: Spontaneous viral clearance.

Table 6. Chi-square or Fisher tests of the response to pegIFN-RBV treatment when we analysed the differences between the genotypes ("/" means that the genotypes have been compared, and "–" that the genotypes are grouped)

Polymorphisms		VR4	VR12	VR24	SVR	SVC
IL28B rs12979860	CC / CT / TT	0.015	0.057	0.098	0.024	–
	CC-CT / TT	0.076	–	–	–	–
	CC / CT-TT	0.006	0.036	0.081	0.008	–
CTLA4 +49	AA / AG / GG	–	–	–	–	0.040
	AA-AG / GG	–	–	–	–	–
	AA / AG–GG	–	–	–	–	0.020
CTLA4 –318	CC / CT / TT	–	–	–	0.074	–
	CC-CT / TT	–	–	–	–	–
	CC / CT-TT	–	–	–	0.061	–
HFE C282Y	N / Hz	–	–	–	–	–
HFE H63D	N / Hz / Ho	–	–	–	–	–
HFE S65C	N / Hz	–	–	–	–	–
LDLr	AA / AG / GG	–	–	–	–	–
	AA-AG / GG	–	–	–	–	–
	AA / AG-GG	–	–	–	–	–
mtDNA Haplogroups	H / J / U / other	–	–	–	–	–

Values indicated the significant probabilities. Lack of signification is indicated as "–". The analyses were done between patients who have responded, or not, at 4 (VR4), 12 (VR12), 24 (VR24) weeks after starting the treatment and patients who have achieved, or not, spontaneous viral clearance (SVC) or sustained viral response (SVR).

Response to pegIFN-RBV treatment. Clinical and virological data were obtained for treatment response at 4, 12, and 24 weeks, and for the SVR endpoint at 48 weeks (Table 5). Of the 55 patients for whom response to treatment data were available, 31 (56.4 %) had not achieved a SVR. As expected, patients infected with HCV genotype 3 had significantly higher SVR rates than those infected with HCV genotypes 1 and 4 ($P = 0.008$).

The relationship between treatment response and the different host polymorphisms is shown in Table 6. The most significant results were obtained for the *IL28B rs12979860* polymorphism. Patients with the CC genotype had higher SVR rates than those with the CT or TT genotypes ($P = 0.008$). The difference was more evident when the CC genotype was compared to the pooled CT and TT genotypes (Fig. 1A). Finally, SVR rates were higher, but not statistically significant, in patients with the T allele in the *CTLA4*–318 SNP.

Patients who did not achieve a SVR (n = 31; 56.4 %) were stratified in two groups. The first consisted of patients who never completely eliminated the virus (non-responders, n = 17; 30.9 %); the second comprised patients with negative viral load at some point after starting treatment but with a later rebound or relapse (relapsers, n = 14; 25.5 %). An analysis of the relationship between the *IL28B* polymorphism genotype and relapse after treatment showed that among patients with the CC genotype only one had never eliminated the virus. Otherwise, over 50 % of the patients who had achieved a SVR had this genotype (CC) ($P = 0.05$) (Fig. 1).

Discussion

The role of host polymorphisms in the response to pegIFN+RBV treatment was studied in a cohort of Majorcan patients with HCV–HIV coinfection. In these patients, we confirmed the strong relationship between the SNP *rs12979860* located upstream of the *IL28B* gene and the response to pegIFN+RBV treatment, as previously reported in HCV mono-infected patients. This relationship points to the importance of this polymorphism in the evolution of HCV infection in HIV coinfected individuals [9]. The allele frequencies detected in our cohort were similar to those of the European-American cohort analyzed by Ge et al. [9]. However, the frequency of the *IL28B rs12979860* C allele was lower in our

cohort than in other Spanish cohorts [23,28], despite the similar allele frequencies of our patients and the controls. Also, in our cohort the frequencies of other SNPs in genes related to the immune system, such as *CTLA4* [5,24,31,36], or to lipid metabolism, such as *LDLr* [19,22,35], were similar to those found in other populations [19,36]. As previously noted, there were no differences in allele frequencies between patient cases and controls, nor were there differences in the mitochondrial haplogroups. By contrast, the H63D mutation in the *HFE* gene was the only SNP that differed in occurrence between patients and controls. While the reason for this difference is unknown, it could be related to the extremely high frequency in Spain of the *HFE* H63D mutation [11].

The average SVR rate of our patient cohort was similar to those usually reported in the literature, although the percentage of patients with SVC was slightly lower and, as expected, SVR rates were lower in patients infected with HCV genotypes 1 and 4 than in those infected with HCV genotype 3 [29,32].

The effect of the *IL28B* genotypes on SVR was significant and more apparent when homozygotes for the favorable *rs12979860* CC alleles were compared with a pooled group comprising heterozygotes and TT homozygotes. In the entire cohort, only one patient with the *IL28B* CC genotype (infected with HCV genotype 3) did not achieve a SVR. By contrast, only one *IL28B rs12979860* TT patient infected with genotype 1 or 4 virus achieved a SVR. The analyses of patients who had relapsed showed that the only patient classified as a non-responder (unable to clear HCV viral load at any time during treatment) had the *IL28B rs12979860* CC genotype. Taken together, these data show that HCV–HIV coinfected patients with the *IL28B* CC genotype were able to achieve an induced HCV clearance with treatment, whereas in non-responders with *IL28B rs12979860* TT, treatment could not "rescue" the non-clearance status.

In patients infected with non-1 HCV genotypes, the role of *IL28B* polymorphisms in predicting SVR is still being investigated [29]. We found a very low frequency of the favorable *IL28B rs12979860* CC genotype in carriers of HCV genotype 4, known to be a difficult-to-treat viral genotype. But whether the virus or the unfavorable *IL28B* genotype precluded SVR in these patients remains to be established. An important finding was that, irrespective of the viral genotype, the *IL28B* CC genotype promoted a rapid response to treatment, as early as 4 weeks after treatment initiation.

There was no relationship between polymorphisms in the *LDLr* gene and the response to treatment. Several reports have associated the *rs14158* GG genotype with a better response [15,26], but in our cohort this genotype was related only to hepatic steatosis, although these results need to be interpreted with caution because of the limited sample size.

Note that all six individuals in the group with spontaneous HCV clearance carried the *CTLA4*+49 G allele. In a previous report, Yee et al. [36] showed that this allele improves the likelihood of a treatment response in patients mono-infected with genotype 1 HCV who were treated with non-pegylated IFN. However, in our cohort of HCV–HIV coinfected patients with HCV genotypes 1, 3, and 4, there was no significant relationship between *CTLA4* and the response to treatment. Other studies have associated the *CTLA4*+49 GG genotype with treatment response, but not with the spontaneous clearance of HCV [24]. Thus, our results add further controversy to this issue, as there was no tendency of a better response to treatment in our patients with the G allele.

Finally, we found no relationship between *HFE* SNPs and any of the variables studied or the response to antiviral therapy. The results reported in the literature for other cohorts are somewhat contradictory. Note that, in our study, all H63D homozygotes, all S65C heterozygotes, and almost all C282Y heterozygotes had undergone liver biopsy previous to their inclusion in our study, which suggests at least the suspicion of liver damage in these patients, although this was not indicated in the pooled fibrosis data.

In summary, the results of this study show the relevance of the CC genotype in the *IL28B* polymorphism regarding the response to pegIFN-RBV treatment in our cohort of HCV–HIVcoinfected individuals from the Balearic Islands. While we identified spontaneous HCV clearance only in *CTLA4*+49 G allele carriers, because of the limited sample size, this association needs to be confirmed in other, larger cohorts.

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Competing interest. None declared.

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