IL28B polymorphisms of both recipient and donor cooperate to influence IFN treatment response in HCV recurrence after liver transplantation, but *IL28B* SNPs of the recipient play a major role in IFN-induced blocking of HCV replication

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SUMMARY

Single nucleotide polymorphisms (SNPs) of the *IL28B* locus are associated with a positive response to pegylated interferon-alpha and ribavirin (pegIFN-alpha/RBV) treatment of HCV-infected patients. This study evaluated the association between SNPs rs12980275, rs12979860 and rs8099917 and treatment outcome of HCV recurrent infection in HCV-positive patients who underwent liver transplant. We aimed to assess to what extent recipient and/or graft donor *IL28B* polymorphisms contribute to HCV clearance after transplantation influencing the response to the antiviral treatment. We found that the allele frequencies in donors were in agreement with the pattern expected in the European population. The frequency of favourable genotypes was significantly lower in recipients than in donors, reasonably because the recipients represented a group of patients affected by chronic Hepatitis C. Our study demonstrated that the positive outcome of the pegIFN-alpha/RBV treatment of HCV recurrence is associated with the co-presence of favourable genotypes of both donors and recipients. However, *IL28B* SNPs of the recipient seem to play a major role in this clinical setting. In particular, homozygosis of rs12979860 favourable genotype in recipients was associated with sustained virological response independently from the donor's genotype. Thus, identification of these SNPs may be useful to predict the response to IFN-based therapy of HCV recurrent infection in liver-transplanted patients.

KEY WORDS: Hepatitis C virus, Liver transplantation, Interleukin *28B*, Polymorphisms, Interferon.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a global public health issue affecting about 3% of the world population. In the majority of cases, the immune system fails to clear HCV replication in the host and the virus establishes a chronic infection that can lead to cirrhosis and hepatocellular carcinoma (Bellanti *et al.,* 2012; Rauch *et al.,* 2010).

The goal of antiviral therapy of HCV infection is virus eradication with sustained virological response (SVR), defined as condition of undetectable HCV RNA in the patient's blood at 24 weeks after the end of the treatment (Bellanti *et al.,* 2012; Kawaguchi-Suzuki *et al.,* 2014).

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Until recently, the standard-of-care for chronic hepatitis C was the treatment with pegylated interferon-alpha and ribavirin (pegIFN-alpha/RBV) (Eurich *et al.,* 2011; Kawaguchi-Suzukiet *et al.,* 2014; Kawaoka *et al.,* 2012). Recently, the use of new direct-acting antiviral agents (DAA) revolutionized HCV therapy producing a major increase in SVR rates (Afdhal *et al.,* 2014; Assis *et al.,* 2012; Lawitz *et al.,* 2014; Naggie, 2012). However, the real efficacy of these drugs in patients with HCV-associated end-stage liver disease has not been established yet and orthotropic liver transplantation (OLT) remains the ordinary treatment for these patients, even if reinfection of the liver allografts by HCV occurs in all cases with pre-OLT HCV-RNAemia (Kawaoka *et al.,* 2012; Vinaixa *et al.,* 2013). The effects of the new DAAs in the post-transplantation HCV recurrence setting have yet to be evaluated.

The response to pegIFN-alpha/RBV treatment is influenced by host and viral factors such as HCV genotype, age, Afro-American ethnicity, gender, stage liver disease, HCV-RNA mutations, and pharmacogenetic factors (Bellanti *et al.,* 2012; Kau *et al.,* 2008; Kawaguchi-Suzuki *et al.,* 2014; Muir *et al.,* 2004).

Genome-wide association studies identified single nucleotide polymorphisms (SNPs) upstream of the *IL28B* gene (rs12980275, rs12979860, and rs8099917) as strongly associated with spontaneous HCV clearance and the efficacy of antiviral therapy with pegIFN-alpha/RBV (Charlton *et al.,* 2011; Ge *et al.,* 2009; Matsuura *et al.,* 2014; Rauch *et al.,* 2010; Tanaka *et al.,* 2009; Thomas *et al.,* 2009; Zhang *et al.,* 2014). This gene is located on chromosome 19 and encodes the interferon-lambda3 (IFNL3) that is known to induce antiviral, immunomodulatory and antitumor activity and the expression of interferon-stimulated genes (Gerley *et al.,* 2012; Honda *et al.,* 2010; Urban *et al,* 2010; Zhang *et al.,* 2014).

Allele A for rs12980275, allele C for rs12979860, and allele T for rs8099917 are defined as *major alleles*. A homozygous haplotype for *major alleles* is associated with a good response to pegIFN-alpha/RBV therapy achieving SVR rates of about 60%, whereas homozygosis for *minor alleles* (allele G for rs12980275, allele T for rs12979860, and allele G for rs8099917) is associated with a SVR of 30% (Charlton *et al.,* 2010; Coto-Llerena *et al.,* 2011).

The clinical setting of post-liver transplantation HCV recurrence in HCV-positive patients is of particular interest for the potential difference between *IL28B/IFNL3* SNPs of the recipient and those present in the donor hepatocytes, which represent the main target of HCV infection after transplantation and, consequently, of antiviral therapy. Our study aimed to analyse the role of recipient and/or donor *IL28B* genotypes in favouring the response to IFN-based therapy of HCV recurrent infection and, consequently, graft survival. We performed a retrospective analysis of a cohort of HCV liver-transplanted patients by testing the distribution of *IL28B* SNPs in pairs of samples of recipient and donor liver DNA. The response to the antiviral treatment was evaluated on the basis of the effect on HCV replication in the host, considering patients with undetectable HCV-RNAaemia at end-of-therapy-response (EOTR) and, among them, patients maintaining HCV aviraemia for more than 24 weeks (SVR).

MATERIALS AND METHODS

Patients

The retrospective study included 57 chronically HCV-infected patients who underwent OLT between July 1999 and September 2013 at ISMETT. Pre-transplantation clinical data were collected for recipients: age, gender, presence of hepatocellular carcinoma, HCV genotype, HCV viral load, anti-HCV therapy regimen, MELD (Model for End-stage Liver Disease) score, and liver biopsy results. Essential information (i.e. age, gender, HCV infection) were collected for donors. The study was approved by the Institutional Research Review Board of ISMETT (IRRB 11/10) and was carried out according to the institutional guidelines.

Assessment of HCV viral load and genotype

HCV-RNA was extracted from plasma samples using High Pure System Viral Nucleic Acid kit (Roche Diagnostics, Manheim, Germany) following the manufacturer's instructions, and HCV-RNA was quantified by real-time PCR using Ampliprep/COBAS TaqMan HCV Test v2.0

(Roche Diagnostics: range 15-1x10^8 IU/ml). HCV genotype was performed using Versant HCV Genotype 2.0 kit (Siemens, Foster City, CA).

Assessment of antiviral therapy outcome

Post-transplantation HCV-induced hepatitis was diagnosed on the basis of the detection of HCV-RNAaemia and >2 stage fibrosis in liver bioptic samples. All patients received pegIFN-alpha/RBV therapy, which was completed or discontinued according to the clinical conditions of the transplanted patients. Reasons for discontinuation of therapy included infections, graft rejection and/or other clinical complications. At the end of the antiviral treatment, HCV-RNAaemia was tested in order to discriminate patients with detectable HCV-RNA (non-responders) and patients with undetectable HCV-RNA indicating a state of EOTR. EOTR patients were then distinguished in those with SVR (aviraemia 24 weeks after the completion of antiviral therapy) or with relapse of HCV replication (detection of HCV RNA after achieving a temporary state of aviraemia at the end of therapy).

***IL28B* genotyping**

Genomic DNA was purified from 200 µL of peripheral blood of recipients using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), and from 5 sections of 5 µm thick paraffin-embedded biopsies of donor's liver using QIAamp DNA FFPE tissue kit (Qiagen), according to the manufacturer's instructions. *IL28B* variants rs12980275, rs12979860, and rs8099917 were genotyped by TaqMan pre-designed SNP Genotyping Assay (Life Technologies, Carlsbad, CA, USA). In particular, 20 ng of genomic DNA was amplified in a volume of 25 µL of PCR reaction containing 1X specific assay mix and 12.5 µL of TaqMan Genotyping master mix (Life Technologies). Genotyping was carried out on 7900HT Fast Real-Time PCR System (Life Technologies) using the following conditions: 95°C for 10 minutes and 60 cycles of amplification (denaturation at 95°C for 15 seconds and annealing/extension at 63°C for 1 minute). Allele discrimination analysis was performed using SDS 2.4 software (Life Technologies).

Statistical analysis

The association between SNPs near the *IL28B* locus and response to antiviral therapy was analysed by Fisher's exact test. All p values were calculated for two-tailed comparisons. p values were defined statistically significant as $p<0.05$, and more statistically significant as $p<0.001$. Statistical association was confirmed by 2-sample z-test, used to compare two sample proportions. Positive predictive value (PPV) and negative predictive value (NPV) were calculated using MedCalc statistical software (version 13.2.2).

RESULTS

Characteristics of the cohort of patients and outcome of antiviral therapy

Fifty-seven chronically HCV-infected patients transplanted between 1999 and 2013 in ISMETT were included in the retrospective study (Table 1). After liver transplantation, all patients experienced a recurrence of HCV infection and were treated with pegIFN-alpha/RBV for 12.3±0.9 months at 16.9±1.8 months after-OLT. At the end of therapy, HCV RNA was detectable in the blood of 22 patients (non-responders) and undetectable in 35 patients (EOTR): 23 of EOTR patients showed SVR whereas 12 of them relapsed HCV infection within 24 weeks

TABLE 1 - *Demographic and clinical characteristics of graft donors and recipients.*

Patients	*Sex*		*Age*[a]	*HCC*	*HCV Genotype*				*Pre-OLT viral load IU/ml*		*Fibrosis score*[b]		*MELD score*[c]	
	M	*F*			*1a*	*1b*	*2a/2c*	*3a*	*>700.000*	*<700.000*	*2*	*3*	*≥20*	*<20*
Donors	40	17	36 (15-76)	-	-	-	-	-	-	-	-	-	-	-
Recipients	49	8	68 (46-75)	33	1	43	1	2	8	9	28	29	36	15

a median value (range); b Fibrosis score 2: scarring has occurred and extends outside the areas in the liver that contains blood vessels; score 3: bringing fibrosis is spreading and connecting to other areas that contain fibrosis; c MELD score ≥20: 19.6% or more of expected mortality within 3 months; score <20: 6% or less of expected mortality within 3 months. HCC, Hepatocarcinoma.

after the end of therapy. Because of serious adverse events caused by IFN-based therapy (leukopenia, thrombocytopenia, anaemia etc.), particularly severe in critical patients such as transplanted patients, pegIFN-alpha/RBV treatment was discontinued prematurely in 21 patients and in 17 cases RBV dose was reduced.

IL28B genotype frequency

Genetic variations in *IL28B* polymorphisms rs12980275, rs12979860, and rs8099917, were evaluated for recipients and donors. As shown in Figure 1, only the allele frequency of donors was in agreement with those of the European population, available in *Allele Frequency Database, ALFRED*, designed to make allele frequency data on human population samples readily available for use by the scientific and educational communities, supported by the U.S. National Science Foundation. Moreover, in the donors, considered as a *representative sample* of the general population, favourable alleles (i.e. C allele in rs12979860) are more frequent than unfavourable alleles (i.e. T allele in rs12979860). By contrast, in recipients the allelic distribution does not appear perfectly consistent with the European population. This result is confirmed by the distribution of individual *IL28B* SNPs, whose frequency was assessed in recipients and in grafts. Our data clearly show that the prevalence of genotypes rs12980275 AA, rs12979860 CC and rs8099917 TT (i.e. homozygosis for the favourable alleles) was less frequently found in recipients than in donors (Figure 2). This result matches the consideration that the lower frequency of homozygosis for favourable *IL28B* genotypes in the recipient could suggest the inability of patients to clear

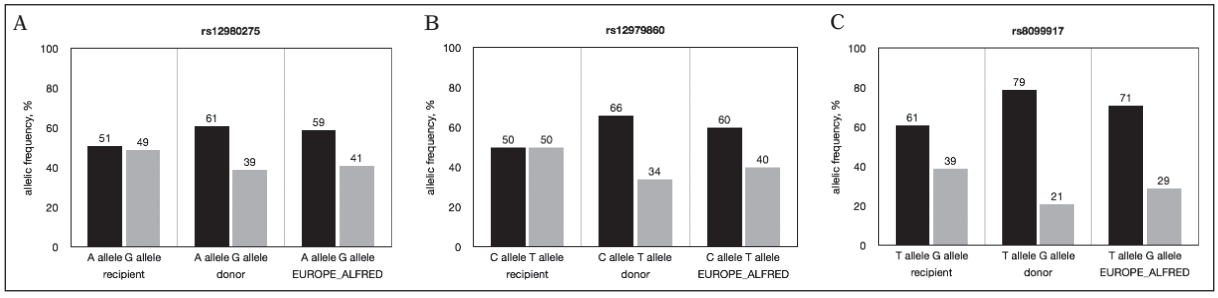

FIGURE 1 - *IL28B allele frequency in recipients and donors compared to the European population. Panel A: rs12980275; panel B: rs12979860; panel C: rs8099917. Favourable alleles: black blocks; unfavourable alleles: grey blocks. ALFRED = Allele Frequency Database.*

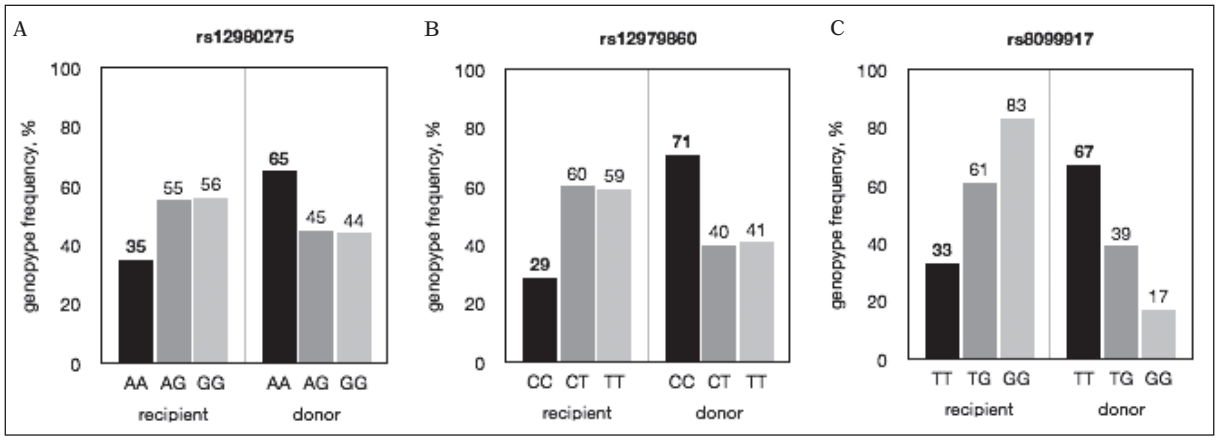

FIGURE 2 - *IL28B genotype distribution among recipients and donors. Panel A: rs12980275; panel B: rs12979860; panel C: rs8099917. Favourable homozygosis: black blocks; heterozygosis: dark grey blocks; unfavourable homozygosis: light grey blocks.*

the infection and consequently, the progression of HCV infection into serious liver damage until liver transplantation.

IL28B genotype and treatment outcome

All HCV-infected patients developed re-infection of the liver graft after transplantation. As reported above, at the end of treatment with pegIFN-alpha/RBV 35 patients presented EOTR and 23 of them SVR. The analysis of the association between *IL28B* SNPs of recipients and donors, and antiviral treatment outcome (Figure 3) demonstrated that the clearance of HCV RNA induced by IFN-based therapy (both permanent as SVR, and temporary as EOTR) is significantly associated with homozygosis of the favourable alleles (rs12980275 AA, rs12979860 CC, rs8099917 TT) in recipients. This result appears particularly remarkable because of the higher frequency of unfavourable genotypes found in recipients, as mentioned above. In particular, rs12979860 CC and rs12980275 AA genotype of recipients were significantly associated with both EOTR and SVR (for rs12980275 AA genotype p=0.037 in EOTR and 0.046 in SVR patients; for rs12979860 CC genotype p=0.004 in EOTR and 0.010 in SVR patients, respectively) (Figure 3). Instead, the rs8099917 TT genotype of recipients was significantly associated with EOTR (p=0.041) but not with SVR. By contrast, *IL28B* polymorphisms of donors did not significantly influence the antiviral treatment response (Figure 3).

We also assessed the effect of the combination of *IL28B* genotypes of recipient and donor on the efficacy of antiviral therapy against HCV recurrence in each transplanted patient (Figure 4). We found that the co-presence of protective favourable homozygous genotypes of both recipients and donors, for rs12979860 and rs12980275 polymorphisms, was associated with a 100% rate of HCV RNA clearance at the end of therapy (EOTR and SVR). However, *IL28B* SNPs of recipient seem to play a major role in this phenomenon. In particular, homozygosis of the rs12979860 CC genotype of the recipient was always associated with a positive antiviral treatment outcome (SVR and EOTR), independently from the genotype of the donor (Figure 4). On the contrary, the favourable donor's genotype is associated with positive response to IFN-based therapy only if combined with the favourable recipient's *IL28B* genotype. We further compared the frequency of the *IL28B* SNP genotypes considered in recipients and donors to analyse the influence of the co-presence of the different SNPs genotypes on the treatment outcome (Table 2). The results showed that the favourable rs12979860 CC genotype was absent in recipients in all non-responder (NR) cases, confirming the positive relation of this genotype with successful antiviral treatment. Inversely, in all SVR/EOTRs no donors presented the unfavourable rs8099917 GG genotype, pointing to the correlation of this genotype with IFN

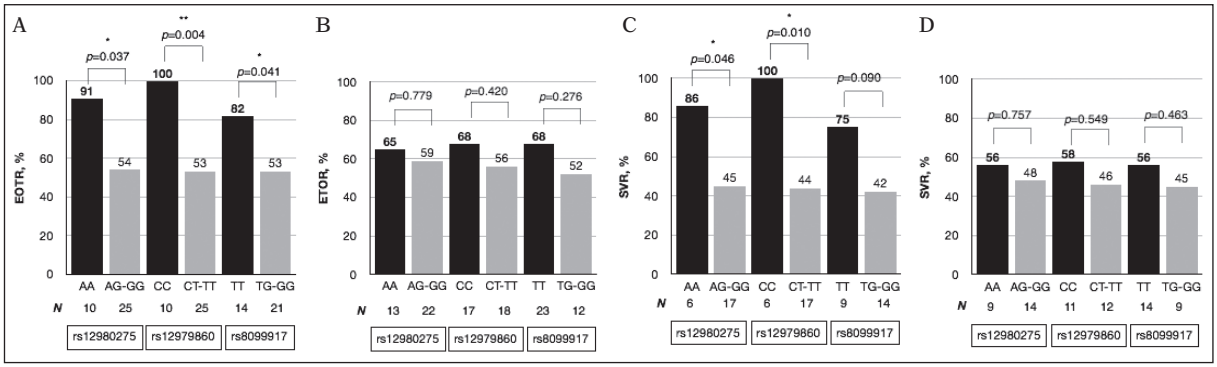

FIGURE 3 - *Antiviral treatment outcomes and IL28B genotypes in recipients and in donors. Panel A: EOTR and recipient's genotype; panel B: EOTR and donor's genotype; panel C: SVR and recipient's genotype; panel D: SVR and donor's genotype. Favourable homozygosis: black blocks; heterozygosis and unfavourable homozygosis: grey blocks. N = number of patients in each group; EOTR = end of treatment viral response; SVR = sustained virological response.*

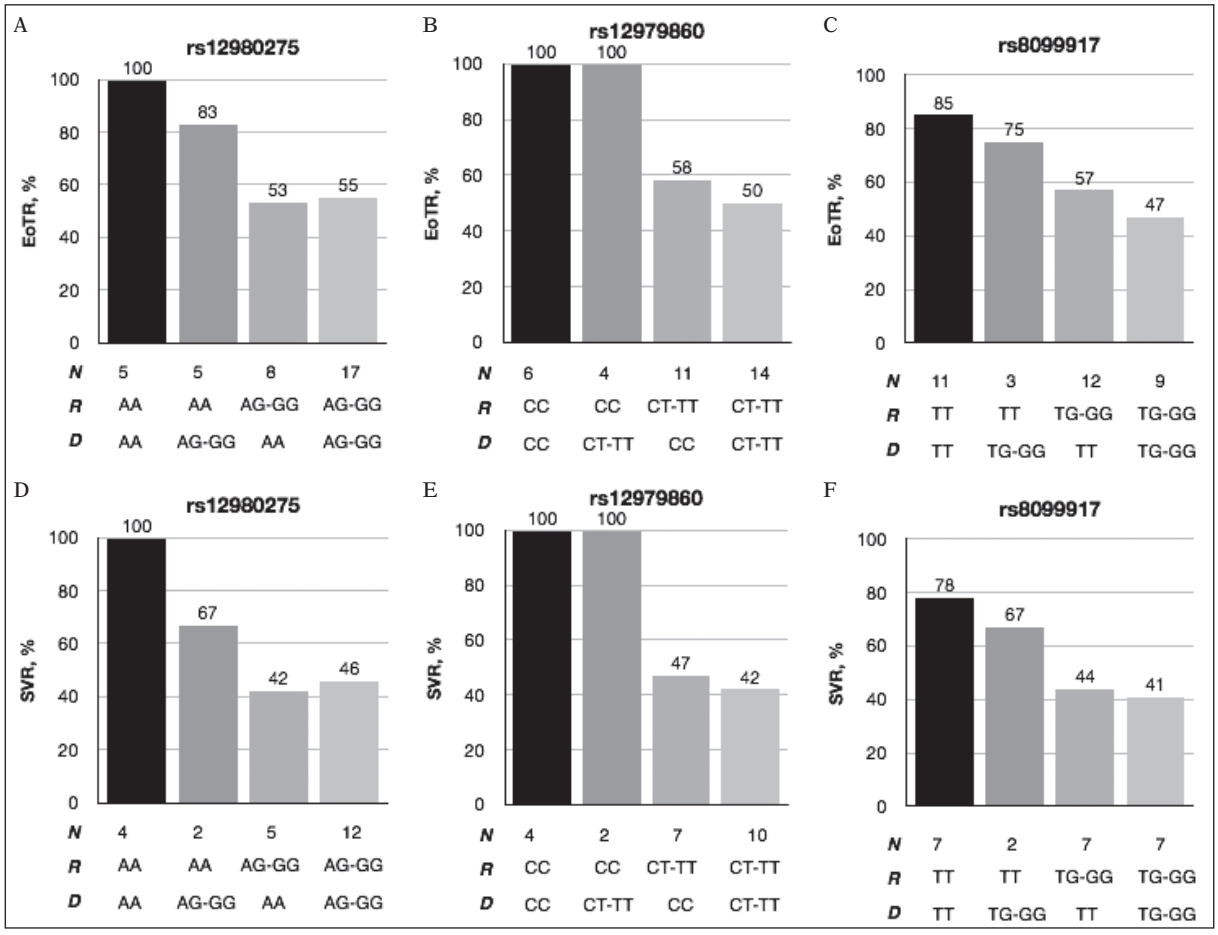

FIGURE 4 - *Effect of the combination of IL28B genotypes of recipient and donor in each liver transplant patient with positive therapy outcome as end-of-treatment-response (panel A: rs12980275; panel B: rs12979860; panel C: rs8099917) and sustained virological response (panel D: rs12980275; panel E: rs12979860; panel F: rs8099917). N = number of patients in each group; EOTR = end of treatment viral response; SVR = sustained virological response; D = donor; R = recipient.*

therapy failure. Moreover, the pattern of association of recipient and donor *IL28B* SNPs with treatment outcome of HCV recurrence after transplantation was similar in patients with SVR and EOTR. Indeed, we found the distribution of rs12980275, rs12979860 and rs8099917 genotypes not significantly different between patients with SVR and relapsers (data not shown). Indeed, it must be pointed out that the treatment of HCV recurrence is administered in critically ill patients treated with immunosuppressive drugs. In addition, four out of 12 relapse patients had not completed IFN-based treatment due to the severity of the side-effects in these type of patients.

Diagnostic tests were performed to assess the accuracy and the predictive value of positive outcome after therapy for favourable genotypes of all the analysed SNPs for both recipients and donors (Table 3). The success of IFN-based treatment rate (EOTR or SVR) in patients with homozygosis of favourable alleles in *IL28B* SNPs was defined as a prediction of successful therapy PPV (positive predictive value). The failure of antiviral therapy in patients without homozygosis of favourable alleles was defined as a prediction of failed therapy NPV (negative predictive value). The presence of favourable genotypes of all SNPs in recipients favours a higher PPV of response than in donors. In particular, for rs12979860 SNP, the PPV was 100%.

TABLE 2 - *IL28B genotype distribution among recipients and graft donors according to IFN-based treatment outcome of HCV recurrent infection*

		SVR	*Relapser*	*NR*
Recipient	rs12980275 AA	26%	33%	14%
	rs12980275 AG	57%	50%	82%
	rs12980275 GG	17%	17%	5%
	rs12979860 CC	26%	33%	0%
	rs12979860 CT	57%	50%	82%
	rs12979860 TT	17%	17%	18%
	rs8099917 TT	39%	42%	14%
	rs8099917 TG	48%	50%	82%
	rs8099917 GG	13%	8%	5%
Donor	rs12980275 AA	39%	33%	32%
	rs12980275 AG	52%	58%	50%
	rs12980275 GG	9%	8%	18%
	rs12979860 CC	48%	50%	36%
	rs12979860 CT	43%	42%	45%
	rs12979860 TT	9%	8%	18%
	rs8099917 TT	61%	75%	50%
	rs8099917 TG	39%	25%	45%
	rs8099917 GG	0%	0%	5%

TABLE 3 - *Positive predictive value (PPV) and negative predictive value (NPV) of IL28B SNPs of recipients and graft donors*

End of treatment response			
		PPV	*NPV*
Recipient	rs12980275 AA	77%	43%
	rs12979860 CC	100%	47%
	rs8099917 TT	82%	48%
Donor	rs12980275 AA	65%	41%
	rs12979860 CC	68%	44%
	rs8099917 TT	68%	48%
Sustained virological response			
		PPV	*NPV*
Recipient	rs12980275 AA	86%	55%
	rs12979860 CC	100%	56%
	rs8099917 TT	74%	58%
Donor	rs12980275 AA	56%	52%
	rs12979860 CC	58%	54%
	rs8099917 TT	56%	55%

DISCUSSION

In recent years, a strong association of specific SNPs upstream of *IL28B*/*IFNL3* gene of the host with spontaneous HCV clearance and response to pegIFN-alpha/RBV therapy of HCV infection has been described (Antonelli *et al.,* 2014; Knapp *et al.,* 2011; Mangia *et al.,* 2013; Tolmane *et al.*, 2012; Zhang *et al.,* 2014). Anti-HCV therapy has recently been rebuilt by the availability of highly effective direct-acting antiviral drugs (Afdhal *et al.,* 2014; Assis *et al.,* 2012; Lawitz *et al.,* 2014; Naggie, 2012). However, in the clinical setting of HCV recurrence in liver-transplanted HCV-positive patients, the possible use of DAAs is still under evaluation and pegIFN-alpha/RBV treatment remains the standard-of-care. Moreover, the possible influence of *IL28B* polymorphisms in IFN-free regimens remains to be elucidated. It must also be considered that IFN-free treatment will be not affordable in many part of the world in the near future. Thus, the analysis of the genetic basis of the response to IFN treatment in the setting of liver transplantation of HCV patients is still of interest.

The liver-transplanted patient is a case of a chimeric condition in which different *IL28B* polymorphisms simultaneously coexist: *IL28B* polymorphism of the host (recipient) and that of the hepatic cells (from the donor), which represent the nearly exclusive target of HCV recurrent infection. Our study was carried out in a cohort of 57 liver-transplanted HCV patients, retrospectively analysing the association between *IL28B* SNPs of recipient and donor of each patient and the outcome of therapy with pegIFN-alpha/RBV of HCV recurrent infection after transplantation. We analysed three SNPs upstream *IL28B* gene - rs12980275 (A/G), rs12979860 (C/T) and rs8099917 (T/G) polymorphisms - that are known to be associated with HCV spontaneous clearance and the antiviral treatment response.

In agreement with other reports (Allam *et al.,* 2013; Bitetto *et al.,* 2013; Charlton *et al.,* 2011; Coto-Llerena *et al.,* 2011; Eurich *et al.,* 2011; Zhang *et al.,* 2014), our results indicate that the presence of the favourable *IL28B* genotype both of recipients and of donors is associated with the development of SVR in post-transplant HCV recurrence. These data appear particularly substantial considering the lower frequency of favourable *IL28B* genotypes found in the recipients (patients affected by chronic C hepatitis evolving to end-stage liver diseases) compared to that of the general population

(like the frequency found in donors), as already pointed out. Evaluating the association of host and donor's liver *IL28* SNPs with SVR after pegIFN-alpha/RBV treatment in each transplanted patient, we showed that homozygosis of favourable alleles in the recipient is significantly associated with positive treatment outcome. Indeed, homozygosis of rs12979860 CC genotype of recipient was associated with HCV RNA clearance (PPV of 100%) after IFN-based treatment even in presence of the non-favourable rs12979860 genotype in the cells of the transplanted liver. Instead, favourable *IL28B* genotypes of the donor were associated with positive response to therapy only if combined with favourable genotypes of the recipient. It must be pointed out that this issue is still matter of debate, since some previous studies concluded that better treatment outcome was related to *IL28B* genotypes of the recipient (Allam *et al.,* 2013; Coto-Llerena *et al.,* 2011; Eurich *et al.,* 2011), some others to *IL28B* genotypes of the donor (Charlton *et al.,* 2011; Kawaoka *et al.,* 2012; Zhang *et al.,* 2014). The evidence of the major role of *IL28B* SNPs of the recipient in influencing the efficacy of pegIFN-alpha/RBV treatment of HCV recurrence after liver transplantation needs further investigation. However, it is important to note that the therapeutic effects of pegIFN-alpha/RBV are not necessarily exerted by interacting directly with the infected cells (mainly donor cells in this setting), but are mediated by activation of immune effector cells of the host (recipient). Indeed strong data suggest a major role of NK cells in the antiviral activity of IFN-based therapy (Dring *et al.,* 2011; Meng *et al.,* 2014; Oliviero *et al.,* 2014). Another aspect to be considered is that following OLT liver tissue undergoes a process of progressive chimerism with the coexistence of cells derived from donors and recipients in the transplanted organ environment (Fukuhara *et al.,* 2010).

Recently it has been shown that the dinucleotide variant rs368234815 located near *IL28B/IFNL3* might represent the strongest host factor for predicting clearance of HCV (Prokunina-*Olsson et al.,* 2013; Krämer *et al.,* 2013; Fernández-Cardillo *et al.,* 2014). In particular, the rs368234815 ΔG genotype causes a frameshift mutation leading to the production of IFN-λ4, which impairs both spontaneous HCV clearance and SVR to IFN therapy (Amanzada *et al.,* 2013; Konishi *et al.,* 2014; O'Brien *et al.,* 2014). Recent data suggest that rs368234815 ΔG genotype may also be relevant for IFN-free regimens with DAAs (Meisner *et al.,* 2014). Thus the IFNλ4 polymorphism in our donors/recipients cohort has to be analysed to further explain our results and obtain insights into the specific role of the IFNL4-ΔG variant in HCV recurrence and treatment after liver transplantation.

In conclusion, our data indicate that the presence of the favourable *IL28B* genotype both of recipients and of donors is associated with a positive response to pegIFN-alpha/RBV treatment of HCV recurrence after liver transplantation of HCV patients.

However, the homozygosis of the protective alleles in recipients - particularly the rs12979860 CC genotype - was the major predictor of HCV RNA clearance at the end of therapy and of the development of SVR in this clinical setting. Since IFN-free treatment may not be affordable for liver transplant patients in the short- term and the possible influence of *IFNL3/IFNL4* polymorphisms on treatment success of regimens with different DAA classes remains to be elucidated, the determination of *IL28B* and *IFNL4* genotypes might be useful for personalizing HCV therapy to optimize cost-effectiveness and avoid side-effects.

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