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Value of IL28B genotyping in patients with HCV-related mixed cryoglobulinemia: results of a large, prospective study

A. Piluso,[1,2,†] C. Giannini,[1,2,†] E. Fognani,[1,2] L. Gragnani,[1,2] P. Caini,[1,2] M. Monti,[1,2] A. Petrarca,[1,2] J. Ranieri,[1,2] T. Urraro,[1,2] E. Triboli,[1,2] G. Laffi[1,2] and A. L. Zignego[1,2] [1]*Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), Department of Internal Medicine, University of Florence, Florence, Italy; and* [2]*IstitutoToscanoTumori (ITT), Florence, Italy*

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SUMMARY. HCV-related mixed cryoglobulinemia (MC) is characterized by clonal expansion of B cells producing a polyreactive natural antibody (rheumatoid factor) and interferon (IFN)-based therapy is the first therapeutic option in mild-moderate MC. Single nucleotide polymorphisms (SNPs) proximal to genes involved in the innate response (IL28B/IFN-λ gene family) are strongly associated with spontaneous and IFN-induced viral clearance in hepatitis C, but no data exist about their role in HCV-positive MC. A large cohort of patients with HCV and MC was studied to evaluate the influence of IL28B genotype on the response to treatment and/or the evolution to MC of HCV infection. The rs12979860/rs8099917 IL28B polymorphisms were analysed in 481 consecutive HCV-positive subjects (250 with MC and 231 without MC, as controls) using real-time PCR and direct sequencing. Hundred and fifteen HCV patients with MC received standard anti-HCV therapy, and the results were evaluated according to the IL28B SNP distribution. Similar IL28B SNPs allele frequencies were recorded for patients and controls. IL28B major allele homozygosis (for both SNPs tested) was tightly correlated with virological and clinical response ($P = 0.002$). A statistically significant association was limited to 'difficult-to-treat' (G1/4) HCV genotypes. The IL28B genotype was a strong independent predictor of response ($P = 0.007$, OR 6.06; CI 1.65–22.22). The IL28B genotype was confirmed to be a useful predictor of response to IFN-based therapy in patients with HCV and MC. The very close correlation between IL28B SNP distribution, virological and clinical response definitively confirmed the key role played by HCV in MC. Conversely, the IL28B genotype does not seem to influence the evolution to MC.

Keywords: hepatitis C virus, interferon-based antiviral treatment, interleukin 28B, mixed cryoglobulinemia.

INTRODUCTION

Hepatitis C virus (HCV) infection is characterized by a high propensity to persist in the host, leading to chronic liver disease, cirrhosis and liver cancer. In the early '90s it was demonstrated that HCV is also a lymphotropic virus [1] and several lymphoproliferative disorders (LPDs) have been associated with HCV infection [2], including B-cell non-Hodgkin's lymphoma (B-NHL) and mixed cryoglobulinemia (MC) [3–14]. MC is an autoimmune and lymphoproliferative disorder characterized by circulating immune-complexes (cryoglobulins: CGs) composed of polyclonal IgGs (including anti-HCV Ig) and mono or polyclonal IgM with rheumatoid factor (RF) activity, sustained by the clonal expansion of RF-B cells [9,15–17]. MC is a prelymphomatous condition whose clinical manifestations – configuring the MC syndrome- are secondary to a systemic vasculitis of the small/medium vessels [18–20]. From the first observations [21,22], a large amount of evidence has been progressively produced about the very close association between MC and HCV infection [16,18,23]. This dramatically modified the therapeutic approach to this disorder. In fact, the observation of a resolution of the MC syndrome in some patients after HCV eradication pushed researchers toward the use of antiviral drugs in addition to the traditional non-etiological therapies (for review see [24]). Starting from the first pioneer studies at the beginning of the '90s [3,25], the antiviral therapy of the HCV-related

Abbreviations: B-NHL, B-cell non-Hodgkin's lymphoma; CGs, cryoglobulins; GWAS, genome-wide association studies; IFN, interferon; LPD, lymphoproliferative disorder; MC, mixed cryoglobulinemia; PBMCs, peripheral blood mononuclear cells; RF, rheumatoid factor; SNP, single nucleotide polymorphism; SOC, standard of care; SVR, sustained virological response.

Correspondence: Anna Linda Zignego, MD, PhD, Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), Department of Internal Medicine, University of Florence, Largo Brambilla 3, 50134 Firenze, Italy. E-mail: a.zignego@dmi.unifi.it
[†]AP and CG equally contributed to this work.

MC followed the evolution of the treatment of chronic HCV infection [18,26] and the attempt at viral eradication using PegIFN plus RBV has recently been indicated as the first-line therapeutic option in patients with mild-moderate HCV-related MC syndrome [24]. However, in patients with MC, this treatment is frequently hampered by contraindications and is followed by several side effects. Consequently, the possibility to predict the outcome before starting therapy would be a precious help and would result in a more tailored approach [27,28].

In 2009 and 2010, several independent genome-wide association studies (GWAS) identified genetic variations in a region in proximity to the interferon (IFN)-λ gene cluster (close to the IL28B gene or IFN-λ3) strongly associated with the HCV clearance [29–33]. The initial studies showed the protective effect of the major allele homozygosis against virus persistence and demonstrated a heterogeneous distribution of the IL28B alleles in different ethnic groups, contributing to explain the differences in treatment response described for particular races [32]. The majority of the studies focused on patients infected by HCV genotype 1 due to the high prevalence and the poor response to the standard of care (SOC) of this viral genotype. In this setting, logistic regression analysis defined IL28B genotyping as the strongest pretreatment predictor of virological response (for review see [34]), while in the 'easy to treat' HCV genotypes (HCV-2 and -3), the predictive value of IL28B genotypes was controversial being not significant [33,35–38] or limited to particular settings of patients according to on-treatment viral kinetics [39].

The importance of IL28B genotyping has been also evaluated in particular categories of HCV-infected patients characterized by modifications of the immune response, namely HIV-coinfected and liver transplanted subjects [32,33,40–43]. However, no data exist about the possible predictive role of IL28B allelic variations in HCV-related LPDs like MC. The pathophysiological characteristics of MC actually do not allow the *a priori* prediction of an identical behaviour as in HCV chronically infected individuals without LPDs. In this light, the predictive value of IL28B polymorphisms in patients with MC-HCV undergoing anti-HCV therapy was evaluated. The possibility that genetic variations in proximity to genes involved in the innate immune response may influence the development of MC was also evaluated. In fact, in MC, the clonally expanded B cells produce polyreactive natural antibodies of the IgM class with RF activity [44], and it has been suggested that RF arising during infections represents an ideal link between innate and acquired immune responses [45,46]. Furthermore, the involvement of TLR activation in the HCV-related disease – particularly of TLR2 by HCV core – has been previously shown, especially in patients with MC [47–49].

PATIENTS AND METHODS

Patients

Four hundred and eighty-one HCV-infected Caucasian patients (228 men/253 women; mean age 60.3 ± 12.7) referred to the outpatient clinic of the Center for the Systemic Manifestations of Hepatitis Viruses (MaSVE), University of Florence, Florence, Italy, prospectively entered the study according to the following inclusion criteria: MC-HCV group (n = 250): presence of a 'definite' MC syndrome, according to previously described criteria [50–52]; HCV group (n = 231): absence of circulating CGs or any other sign/symptom of MC. Patients without a definite MC syndrome, with only the presence of CGs and/or other stigmata of MC or any other autoimmune/LPD, were excluded from the study. Clinical and laboratory characteristics at baseline of the 481 patients with HCV are summarized in Table 1. A subgroup of the MC-HCV (n = 115, Table 2) with MC syndrome and without treatment contraindications completed antiviral treatment (Peg-IFN plus RBV), following current international SOC [28,53] and a 6-month post-treatment follow-up. All patients had mild to moderate MC syndrome, in particular, no patients at baseline had severe renal impairment (creatinine clearance >50 mL/min in all cases). In no case, corticosteroid or other immunosuppressive agents were administered during the study. Most of these patients with MC also belonged to a prospective study aimed at evaluating, in a long-term follow-up, the effects of HCV persistence or clearance after anti-HCV SOC in patients with MC (Gragnani *et al.*, manuscript in preparation). Diagnosis of HCV infection and HCV-related chronic hepatitis was performed according to previously described criteria [52,54,55].

A complete MC clinical response was defined as improvement in all baseline clinical manifestations and a partial response as improvement in at least half of the baseline clinical symptoms. All other patients were classified as clinical nonresponders. Criteria for both clinical and virological response have been previously described in detail [28,50,52,56,57]. All patients provided informed consent in accordance with the Principles of the Declaration of Helsinki and approved by the local Ethics Committee.

Cell isolation and DNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh anticoagulated blood by gradient precipitation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) according to manufacturer's instructions. After the second wash, the cells were counted and stored at −80 °C. Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN Inc, Valencia, CA, USA) according to the manufacturer's instructions.

Table 1 Main clinical and laboratory data of 481 Hepatitis C virus-positive patients with (MC-HCV) or without (HCV) mixed cryoglobulinemia syndrome

	HCV (n = 231)	MC-HCV (n = 250)	P value
Mean age (years)	56.8 ± 12.9	63.4 ± 11.7	<0.0005
Sex (male/female)	148/83	80/170	<0.0005
Histology			0.003
Chronic Hepatitis	189	176	
Cirrhosis	42	74	
ALT (ULN)	3.85 ± 2.4	3.43 ± 2.2	ns
Viral titer (IU/mL × 10^6)	2.3 ± 2.9	2.8 ± 1.72	ns
HCV genotype			ns
1	119 (51.5%)	134 (53.6%)	
2	67 (29.0%)	82 (32.8%)	
3	34 (14.7%)	28 (11.2%)	
4	11 (4.8%)	6 (2.4%)	
Mean cryocrit (%)	0	8.2 ± 7.2	<0.0005
Mean C3* (mL/dL)	115.3 ± 63.2	104.5 ± 61.5	ns
Mean C4† (mL/dL)	91.6 ± 45.7	10.5 ± 11.3	<0.0005
Mean rheumatoid factor‡ (IU/mL)	16.7 ± 8.0	380.5 ± 292.4	<0.0005
IL28B (rs12979860)			ns
C/C (%)	91 (39.4)	99 (39.6)	
C/T (%)	110 (47.6)	113 (45.2)	
T/T (%)	30 (13.0)	38 (15.2)	
Presence of T allele (%)	140 (60.6)	151 (60.4)	
IL28B (rs8099917)			ns
T/T (%)	138 (59.7)	152 (60.8)	
G/T (%)	79 (34.2)	76 (30.4)	
G/G (%)	14 (6.1)	22 (8.8)	
Presence of G allele (%)	93 (40.3)	98 (39.2)	

ns, not significant; ALT, alanine aminotransferase; ULN, upper limit of normal. Results are presented as mean ± standard deviation. *Complement C3, normal values: 83–177 mL/dL. †Complement C4, normal values: 20–150 mL/dL. ‡Rheumatoid Factor, normal values: <25 IU/mL.

Table 2 Main clinical and laboratory data of 115 HCV patients with mixed cryoglobulinemia syndrome resulting sustained virological responders (SVR) or non-responders (NR)/relapsers after antiviral therapy

	SVR (n = 61)	NR/Relapsers (n = 54)	P value
Mean age at treatment (years)	48.2 ± 10.3	51 ± 9.9	ns
Sex (male/female)	22/39	20/34	ns
ALT* (ULN)	3.22 ± 2.5	3.23 ± 2.1	ns
Histology*			<0.0005
Chronic hepatitis	55	31	
Cirrhosis	6	23	
HCV mean titer*(IU/mL × 10^6)	2.19 ± 4.05	3.38 ± 3.67	ns
HCV genotype			<0.0005
1–4	17 (30.5%)	41 (79.3%)	
2–3	44 (69.5%)	13 (20.7%)	
Mean cryocrit* (%)	7.3 ± 5.8	7.1 ± 6.1	ns
Mean C4† (mL/dL)	121.8 ± 52.8	124.5 ± 60.6	ns
Mean C3‡ (mL/dL)	12.8 ± 11.4	13.1 ± 12.1	ns
Mean rheumatoid factor§ (IU/mL)	388.5 ± 205.4	403.5 ± 198.2	ns

ns, not significant; NR, non-responders; SVR, sustained viral responders; ALT, alanine aminotransferase; ULN, upper limit of normal. Results are presented as mean ± standard deviation. *At baseline. †Complement C4, normal values: 20–150 mL/dL. ‡Complement C3, normal values: 83–177 mL/dL. §Rheumatoid Factor, normal values: <25 IU/mL.

IL28B genotyping

IL28B genotyping was performed using a specific custom TaqMan SNP-Genotyping Assay (Single Nucleotide Polymorphism (SNP): rs12979860 and rs8099917; Applied Biosystem, Foster City, CA, USA) based on allele-specific dual-labelled probes on a Rotor Gene 6000 (Corbett Research, Sidney, Australia). Amplicon sequencing was used to validate the genotyping techniques. All genotyping results were consistent with the Hardy–Weinberg equilibrium.

Statistical analysis

Data are expressed as the mean ± SD. Quantitative variables were analysed using the unpaired Student's *t*-test and the unpaired Wilcoxon's test when necessary. Categorical variables were analysed with Pearson's χ^2 test and Fisher's exact test when necessary. All tests were two-sided at a 0.05 significance level. Predictive factors of response were determined by a multivariate logistic regression analysis. Analyses were performed by the Stata v.9.0 (StataCorpLP, College Station, TX, USA).

RESULTS

The MC-HCV group (250 patients with 'definite' HCV-related MC syndrome) and the HCV group (231 patients with HCV chronic infection without any sign or symptom of MC or other LPD) did not differ regarding viremia titres or viral genotype distribution, whereas, as expected, female sex was significantly more represented in the MC-HCV group ($P < 0.0005$), as well as older age ($P < 0.0005$) and liver cirrhosis ($P = 0.003$) (Table 1). The allele distribution of the IL28B SNPs (rs12979860 and rs8099917) was similar in patients with or without MC with the C/C homozygosis (rs12979860) and the T/T genotype (rs8099917) in about 40% and 60% of patients, respectively, in both groups (Table 1).

In 115 treated patients with MC-HCV, a clear correlation between the distribution of the IL28B alleles and both the clinical and virological response to the IFN-based therapy was noticed (Table 2). Among these, only 2 of the 61 patients (3.2%) experiencing a sustained virological response (SVR) maintained a definite MC syndrome (even if milder than the pretreatment one: partial clinical response). All other patients (not achieving SVR = virological nonresponders or relapsers) resulted clinical non-responders at the end of follow-up, in spite of a transient MC improvement in some cases ($P < 0.0005$). In more detail, patients that experienced a consistent improvement in all baseline clinical manifestations, justifying the definition of 'complete clinical responders', included subjects with a complete disappearance of all the symptoms and biohumoral signs of MC syndrome (corresponding to the majority of patients with SVR: 35/61; 57%) and patients in which the isolated persistence of one or more biohumoral signs or symptoms was noticed at the end of follow-up (26/61; 43% SVR patients). In Table 3, the prevalence of isolated signs/symptoms persisting in these latter 26 patients is detailed. The analysis of IL28B genotypes in treated patients showed similar results for both rs12979860 and rs8099917 SNPs tested. For this reason, only data corresponding to rs12979860 are described in the tables and figures. Homozygosis for the major IL28B allele (C/C for rs12979860 and T/T for rs8099917, data not shown) was significantly associated with a sustained clinical response (Fig. 1, panel a; $P = 0.002$). As outlined previously, only 2 of 61 patients with MC achieving SVR did not obtain complete clinical response; of these, one bore homozygosis for the major IL28B allele and the other heterozygosis. On the whole, the IL28B genotype distribution was closely related to both the clinical and the virological response in the total number of treated patients with MC-HCV. When the different HCV genotypes were considered, a significant statistical association between homozygosis for the major IL28B allele and a sustained response was evident only in patients with MC-HCV infected by 'difficult-to-treat' HCV genotypes (HCV-1 and 4; $P = 0.015$) (Fig. 1, panel b and c). The multivariate logistic regression analysis indicated the presence of homozygosis for IL28B major allele as a strong independent predictor of MC response ($P = 0.007$, OR 6.06; 95% CI 1.65–22.22; Table 4).

DISCUSSION

In the present study, for the first time, the IL28B polymorphic variants were analysed in patients with HCV-related

Table 3 Principal MC manifestations (clinical and biohumoral) diagnosed before treatment and at the end of a 6-month follow-up in the 61 patients with MC-HCV who achieved a sustained virological response

MC manifestations	Pretreatment (%)	End of F-Up (%)
Clinical		
Purpura	48 (78.6)	2 (3.3)
Arthralgias	51 (83.6)	7 (11.4)
Weakness	55 (90.1)	13 (21.1)
Neuropathic symptoms	46 (75.5)	8 (13.1)
Renal involvement	9 (14.7)	0
Skin ulcers	8 (13.1)	0
Sicca syndrome	28 (45.9)	11 (18.0)
Biohumoral		
Cryoglobulins	61 (100)	2 (3.3)
Rheumatoid Factor*	59 (96.7)	19 (31.1)
Reduced C4†	53 (86.8)	6 (9.8)

MC, mixed cryoglobulinemia. *Rheumatoid Factor: elevated rheumatoid factor levels upper the normal values (<25 IU/mL). †Reduced C4: Complement C4 levels below the normal values (20–150 mL/dL).

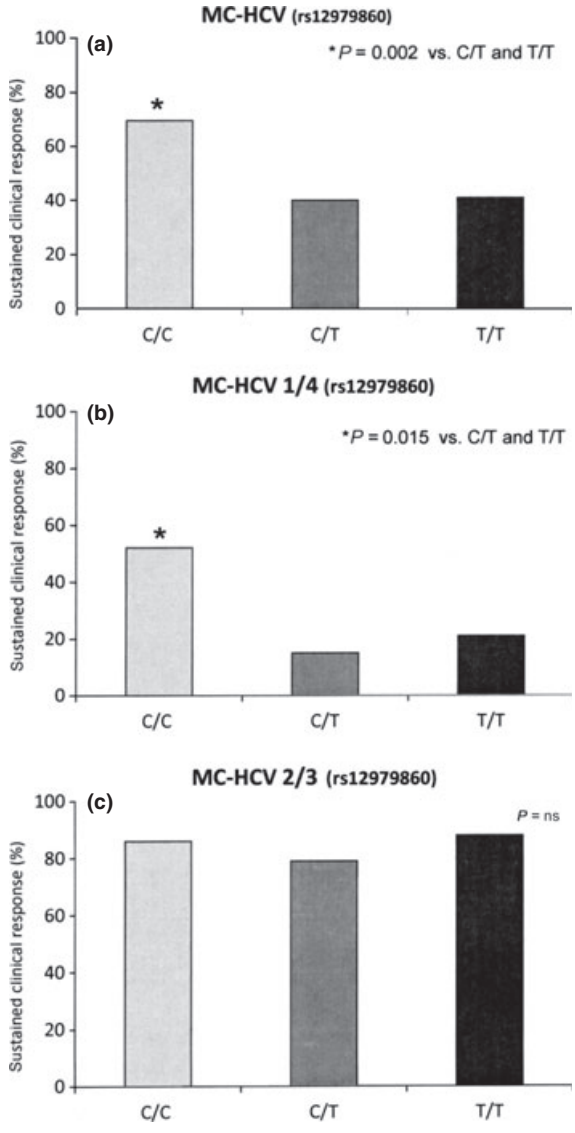

Fig. 1 Rate of HCV patients with mixed cryoglobulinemia (MC) syndrome achieving a sustained clinical response according to the IL28B (rs12979860) genotype and the HCV genotype. Panel (a): total population (n = 59); Panel (b): patients with MC infected by HCV genotype 1 and 4 (n = 17); Panel (c): patients with MC infected by HCV genotype 2 and 3 (n = 44). NS, not significant.

MC. The IL28B genotype distribution was a strong pre-treatment predictor of response to IFN-based, SOC therapy, in patients harbouring the most frequent HCV 1 genotype. Furthermore, the IL28B genotype did not appear as a genetic factor influencing the evolution of HCV infection to MC syndrome.

In the field of HCV-related chronic liver disease, independent GWAS have clearly shown that genetic variations in the IL28B gene (interferon-λ3) determine the outcome of IFN-α-based therapy [29–32] as well as the likelihood of

Table 4 Factors associated with sustained virological responders by logistic regression model in 115 patients with MC-HCV

Factors	OR	95% CI	*P* value
Age (<50 *vs* >50 year old)	4.50	1.18–17.10	0.027
Gender (female *vs* male)	1.78	0.50–6.29	0.368
HCV genotype (2/3 *vs* 1/4)	11.25	3.20–39.46	0.000
Liver histology (chronic hepatitis *vs* cirrhosis)	9.60	2.28–40.49	0.002
IL28B rs12979860 (C/C *vs* C/T and T/T)	6.06	1.65–22.22	0.007
Baseline viral load (<500 000 IU/mL *vs* >500 000 IU/mL)	4.11	1.13–14.92	0.031

spontaneous clearance following acute HCV infection [58,59]. Interestingly, a consistent difference in the distribution of IL28B alleles in different regions of the world was observed, with consistent discrepancies between East Asia and South Europe [29,32] regions also characterized by different prevalence of HCV-related MC [60]. In consideration of the fact that the exact mechanisms involved in the development of MC during the course of chronic HCV infection are still unknown and that the IL28B polymorphisms represent variants of genes coding for immunity-related cytokines, it is impossible to *a priori* assess a predictive value of this genetic marker similar to what is observed in uncomplicated HCV-related chronic liver disease. Indeed, the relevance of cytokines and chemokines pattern modifications in HCV-related MC pathogenesis has been reported by several studies [61–64]. No data concerning MC-HCV are still available, possibly due to the difficulty in recruitment of a sufficiently large population of patients with this rare disease. For similar reasons, it is not possible to exclude that genetic variations in the IL28B gene may somewhat influence the predisposition of patients with chronic HCV infection to evolve to MC syndrome. In this light, in the present study, a large population of patients with 'definite' HCV-related MC syndrome was compared with chronic HCV patients without any signs or symptoms of MC (or other LPD), to evaluate the distribution of the IL28B SNPs (rs12979860 and rs8099917). The allele frequencies found in MC-HCV and HCV groups for both SNPs were consistent with previous reports in Caucasian populations from Europe [39,65] showing a correct genotyping, as also confirmed by the consistency with the Hardy–Weinberg equilibrium. No significant differences were observed in the studied populations. Consequently, our results do not support the hypothesis that genetic variations in proximity to IL28B genes influence the development of MC. Concerning the predictive value of IL28B polymorphism in the context of IFN-based therapy, it is of note that this treatment has now reached a key position in

the therapeutic management of MC-HCV. In several studies, although performed with different therapeutic schedules and consequent variable outcomes, it was shown that IFN-based MC treatment was able to persistently improve or resolve the MC clinical picture in a consistent proportion of patients and that this was related to the virological response (for review [3]). Subsequently, anti-HCV therapy with the 'SOC' combined Peg-IFN plus RBV treatment is generally considered the first therapeutic option in patients with MC-HCV without contraindications or severe disease [18,24]. In the present study, a large group of treated patients with MC-HCV was tested for the IL28B polymorphisms. A clear correlation between the distribution of the IL28B alleles and both the clinical and virological response was shown. This was allowed by a strong association between clinical and virological response to the antiviral treatment that fully confirmed previous observations [24]. This very close correlation between IL28B SNPs distribution, virological and clinical response definitively confirmed the key role played by HCV in MC.

In more detail, considering the total population of treated patients with MC, a significant association between homozygosis for the major IL28B allele and both a sustained clinical and virological response was shown and the multivariate logistic regression analysis indicated that the major allele homozygosis for IL28B is a strong, independent predictor of MC syndrome response. However, when the different HCV genotypes were considered, a significant statistical association was evident only in MC-HCV patients with the most frequent and 'difficult-to-treat' HCV genotypes (HCV-1 and 4) [28,66,67]. This especially accounts for the clinical utility of additional predictive markers to be used in the management of HCV type 1/4 patients showing a symptomatic, frequently invalidating condition, like MC syndrome, for which alternative therapies are available [24]. The observation of a more consistent prognostic value of IL28B genotypes in HCV G1/4 than in HCV G2/3 infection was previously made in patients with hepatitis C [33,36]. Further studies will determine whether, in analogy with what has been observed in cases of chronic hepatitis, the determination of IL28B genotypes may help to tailor therapy for patients with easy-to-treat HCV G2/3, but without rapid virological response [39].

In conclusion, this study is the larger so far performed on patients with MC treated with traditional SOC anti-HCV therapy with Peg-IFN plus ribavirin and the first analysing the role played by genetic IL28B polymorphisms. The results of this study for the first time show that the IL28B genotype is a strong pretreatment predictor of response to IFN-based therapy, also in patients with this complex HCV-related autoimmune/LPD. In this setting, the determination of the IL28B polymorphism appears as a noninvasive, potentially very useful tool especially relevant in patients with the most frequent and difficult-to-treat HCV genotype (HCV G1). Consequently, it is expected that the determination of the IL28B genotype will allow a more personalized therapeutic approach to this complex condition. This would be of particular utility, especially when considering the side effects of therapy as well as its costs.

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CONFLICT OF INTEREST

The authors declare the absence of any conflict of interest.

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