Vitamin D Binding Protein Gene Polymorphisms and Baseline Vitamin D Levels as Predictors of Antiviral Response in Chronic Hepatitis C

Edmondo Falleti,[1] Davide Bitetto,[1] Carlo Fabris,[1] Giovanna Fattovich,[2] Annarosa Cussigh,[1] Sara Cmet,[1] Elisa Ceriani,[3] Ezio Fornasiere,[1] Michela Pasino,[2] Donatella Ieluzzi,[2] Mario Pirisi,[3,4] and Pierluigi Toniutto[1]

Vitamin D deficiency seems to predict the unsuccessful achievement of sustained viral response (SVR) after antiviral treatment in hepatitis C virus (HCV) difficult-to-treat genotypes. Vitamin D binding protein (GC) gene polymorphisms are known to influence vitamin D levels. This study was performed to assess whether the interaction between basal circulating vitamin D and the GC polymorphism plays a role in influencing the rate of antiviral responses in patients affected by chronic hepatitis C. In all, 206 HCV patients treated with a combination therapy of pegylated (PEG)-interferon plus ribavirin were retrospectively evaluated. GC rs7041 G>T, GC rs4588 C>A, and IL-28B rs12979860 C>T polymorphisms were genotyped. Frequencies of GC rs7041 G>T and rs4588 C>A polymorphisms were: G/G = 64 (31.1%), G/T = 100 (48.5%), T/T = 42 (20.4%) and C/C = 108 (52.4%), C/A = 84 (40.8%), A/A = 14 (6.8%). Patients were divided into those carrying ≥3 major alleles (wildtype [WT]+: G-C/G-C, G-C/T-C, G-C/G-A, N = 100) and the remaining (WT−: G-C/T-A, T-A/T-C, T-A/T-A, T-C/T-C, N = 106). Four groups were identified: vitamin D ≤20 ng/mL and WT−, vitamin D ≤20 and WT+, vitamin D >20 and WT−, vitamin D >20 and WT+. In difficult-to-treat HCV genotypes the proportion of patients achieving SVR significantly increased with a linear trend from the first to the last group: 6/25 (24.0%), 9/24 (37.5%), 12/29 (41.4%), 19/29 (65.5%) ($P = 0.003$). At multivariate analysis, having basal vitamin D >20 ng/mL plus the carriage of GC WT+ was found to be an independent predictor of SVR (odds ratio 4.52, $P = 0.015$). *Conclusion*: In difficult-to-treat HCV genotypes, simultaneous pretreatment normal serum vitamin D levels and the carriage of GC-globulin WT isoform strongly predicts the achievement of SVR after PEG-interferon plus ribavirin antiviral therapy. (HEPATOLOGY 2012;56:1641-1650)

New direct hepatitis C virus (HCV) protease inhibitors (boceprevir and telaprevir) have recently been introduced to be used in combination with pegylated (PEG)-interferon and ribavirin in the treatment of chronic hepatitis C.[1,2] Triple antiviral therapy was associated with a significant increase in sustained viral response (SVR) in patients infected by HCV genotype 1. Nevertheless, the need of interferon sensitiveness, the frequent low tolerability, and the occurrence of multiple drug-to-drug interactions will probably limit the use of these molecules to selected populations.[3] Therefore, affordable pretreatment predictors of SVR to standard therapy will be useful to select naïve patients in whom side effects and costs of triple therapy could be avoided without reducing the rate of SVR achievement.

Several predictors of successful treatment of chronic hepatitis C have been identified.[4,5] Factors related to the virus appear to be carefully characterized: infection

Abbreviations: BMI, body mass index; cEVR, complete early viral response; EOT, end of treatment viral response; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immune-deficiency virus; HOMA, homeostasis model assessment; IL-28B, interleukin 28B; PEG, pegylated; RVR, rapid viral response; SVR, sustained viral response; WT, wildtype.

From the [1]Department of Medical Sciences Clinical and Experimental, Medical Liver Transplantation Unit, Internal Medicine, University of Udine, Italy; [2]Gastroenterology Clinic, Department of Medicine, Azienda Ospedaliero-Universitaria, University of Verona, Italy; [3]Department of Clinical and Experimental Medicine, Università del Piemonte Orientale "A. Avogadro", Novara, Italy; [4]Interdepartmental Research Centre for Autoimmune Diseases (IRCAD), Novara, Italy.

Received March 11, 2012; accepted May 7, 2012.

Mario Pirisi was supported in part by grants from the Ricerca Sanitaria Finalizzata Program, Regione Piemonte, Italy.

by HCV genotypes 2-3 confers a much greater possibility of a successful treatment than infection by genotypes 1 and 4; other predictors are basal HCV viral load and HCV genetic variability. Host predictors are classifiable into genetic and nongenetic. Among the latter, a higher degree of liver inflammation and fibrosis and lower serum cholesterol values appear to have a major role.[4,6] Among the former, the major genetic determinant of HCV clearance has been identified in interleukin 28B (IL-28B) polymorphisms.[7,8] Possessing the IL-28B rs12979860 C/C genotype confers a greater advantage for HCV clearance with standard therapy than the carriage of one or two T alleles.[7]

Serum vitamin D concentration has been very recently proposed as a novel predictor of HCV clearance. In patients with genotype 1 chronic hepatitis C, under standard PEG-interferon plus ribavirin treatment, basal serum 25-hydroxylated vitamin D concentration was found to be an independent predictor of viral clearance.[9] Moreover, normal basal vitamin D values were associated with a better antiviral response to therapy than the presence of vitamin D deficiency in patients with the same IL-28B genotype.[10] Finally, vitamin D supplementation was associated with an increased rate of SVR in patients with HCV genotype 1 chronic hepatitis.[11] Based on several recent reports,[12] vitamin D appears to possess important immune-modulator effects that may explain these observations.

Multiple factors are implicated in regulating vitamin D levels and activity.[13] GC-globulin is the main serum vitamin D binding protein; it is polymorphic and characterized in Caucasian populations by a great variability at two loci (rs7041 and rs4588). The carriage of the minor alleles has been associated with lower circulating vitamin D levels.[14] The influence of these polymorphisms on vitamin D levels in patients with chronic hepatitis C and on the rate of antiviral response to standard therapy for this infection has never been investigated.

This study was performed to assess whether the interaction between basal circulating vitamin D and the polymorphism of its transporter (vitamin D axis) plays a role in influencing the rate of antiviral responses in patients affected by chronic hepatitis C.

Patients and Methods

Patients. The study population included a total of 206 consecutive, treatment-naïve Caucasian hepatitis C patients who were retrospectively evaluated. All the patients received antiviral treatment at one of three academic centers in northern Italy (the Medical Liver Transplantation Unit of the University of Udine [N = 71, 34.5%], the Department of Gastroenterology of the University of Verona [N = 88, 42.7%], and the Department of Clinical and Experimental Medicine, University of Piemonte Orientale in Novara [(N = 47, 22.8%]) from September 2005 to October 2009. Chronic hepatitis C infection was defined by the presence of antihepatitis C virus antibodies, serum HCV RNA positivity, and the persistent elevation of alanine aminotransferase (ALT) for at least 6 months. In addition, 142 patients had had a liver biopsy performed within the 6 months preceding the start of antiviral therapy. Exclusion criteria were: decompensated liver cirrhosis (Child-Pugh score >6), the presence of hepatocellular carcinoma (HCC), HIV coinfection, HBV coinfection, autoimmune liver disease, genetic liver disease (i.e., Wilson's disease, hemochromatosis), concomitant use of drugs known to affect serum vitamin D concentration, and active intravenous drug use. The main clinical and demographic characteristics of the studied population are reported in Table 1. The study was conducted according to the principles of the Declaration of Helsinki and approved by the hospital Institutional Review Board and Ethical Committee. All the patients signed a written informed consent to participate into the study. The following variables were recorded prior to treatment: age, sex, body weight, height and body mass index (BMI), calculated on the basis of body weight in kilograms and height in meters. Alcohol intake was evaluated by a questionnaire and quantified in grams per day. An overnight fasting blood sample was drawn to determine the baseline blood tests, including HCV RNA quantification, using real-time polymerase chain reaction (PCR) (TaqMan, Roche, Basel, Switzerland), and HCV genotype, detected using the InnoLipa genotyping kit (Innogenetics, Zwijndrecht, Belgium). In all, 119 healthy blood donors were used as controls. They were 73 males (61.3%) with a median age of 50 years (range

Address reprint requests to: Pierluigi Toniutto, MD, Internal Medicine, Medical Liver Transplantation Unit, University of Udine, Italy. E-mail: pierluigi.toniutto@uniud.it; fax: +390432559490.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.25848
Potential conflict of interest: Nothing to report.

Table 1. Characteristics of the Studied Population at Baseline (N=206)

Age, years		48 (17-77)
Male gender		106 (51.5%)
Body mass index kg/m^2		24 (16-39)
HCV genotypes	1	92 (44.7%)
	2	61 (29.6%)
	3	38 (18.4%)
	4-5	15 (7.3%)
HCV RNA (x 10^3 IU/mL)		700 (3.38-13000)
Cholesterol mg/dL		170 (72-327)
ALT IU/mL		68 (12-428)
γGT IU/mL		38 (6-875)
HOMA		2.0 (0.2-13.3)
Use of PEG-interferon α-2b		145 (70.4%)
Grading pretreatment*		2 (0-9)
Staging pretreatment*		2 (0-6)
Alcohol consumption g/day		0 (0-50)

Continuous variables are presented as median (range), while categorical variables are presented as frequencies (%).

ALT, alanine aminotransferase; γGT, gamma-glutamyl transpeptidase; HOMA, homeostasis model assessment.

*Available in 142 patients.

25-65) and with a median BMI of 24 (range 18-33) kg/m^2. No significant difference was observed between patients and controls in gender ($P = 0.084$), age ($P = 0.944$), or BMI ($P = 0.112$).

Vitamin D Assay. A pretreatment blood sample for the 206 patients and a standard 8 hours fasting blood sample for the 119 controls was collected, separated, and stored at −80°C until use. To quantify 25-OH vitamin D serum levels the DiaSorin 25-OH vitamin D chemoluminescent immunoassay on a Liaison automatic analyzer (DiaSorin, Stillwater, MN) was used.

Molecular Biology. Genotyping for the IL-28B rs12979860 C/T, for GC rs7041 T/G (Asp432Glu), and for GC rs4588 C/A (Thr436Lys) polymorphisms was performed by PCR-based restriction fragment length polymorphism assay. Genomic DNA was extracted from whole blood samples by means of the QIAamp DNA blood mini kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. For IL-28B rs12979860 C/T a 145-basepair (bp) product was obtained with the forward primer 5′-GCCTGTCGT GTACTGAACCA-3′ and the reverse primer 5′-AGGCTCAGGGTCAATCACAG-3′. For GC rs7041 T/G and GC rs4588 C/A polymorphisms a 125 bp amplicon was obtained with the 5′-TGGTTTTTCA-GACTGGCAGA-3′ forward and 5′-GGAGGTGAG TTTATGGAACAGC-3′ reverse primers. All PCR amplifications were carried out in a total volume of 10 μL. Samples containing 10 ng of genomic DNA were subjected to 40 cycles of denaturation (95°C for 30 sec), annealing (60°C for 30 sec), and elongation (72°C for 30 sec) using a Techne TC-5000 thermal cycler. In a total volume of 20 μL, amplified DNA (10 μL) was digested overnight with 2 units of restriction endonucleases using the buffers and temperatures recommended by the manufacturer. Hpy166II restriction endonuclease was used for IL-28B rs12979860 C/T, HaeIII for GC rs 7041 T/G, and BtgI for GC rs4588 C/A polymorphisms (all endonucleases from New England Biolabs, Hitchin, UK). The restricted fragments were 135 +10 bp for the C allele and 105+30+10 bp for the T allele of IL-28B rs12979860, 125 bp for the T allele and 89+36 for the G allele of GC rs7041, 125 bp for the A allele and 71+54 bp for the C allele of GC rs4588. The fragments were resolved by electrophoresis on a 3.5% agarose gel after staining with ethidium bromide. Haplotype reconstruction from patients genotypes and inferred phased diplotype calculation was performed by means of the ARLEQUIN integrated software package for population genetics. On the basis of the diplotypes obtained and taking into account the number of major alleles present in the two loci studied, patients were divided into two groups: WT+ accounted for by those carrying three or four major alleles (G-C/G-C, G-C/T-C, G-C/G-A, N = 100) and WT− accounted for by the remaining patients (G-C/T-A, T-A/T-C, T-A/T-A, T-C/T-C, N = 106). This categorization was more capable of discriminating patients with low or normal vitamin D values.

Histology. As mentioned above, 142 out of 206 patients (68.9%) underwent a liver biopsy before starting therapy. Grade and stage were scored according to the Ishak system.[15]

Antiviral Therapy Schedule and Outcomes. All patients were treated with a combination therapy of PEG-interferon plus ribavirin. In all, 145 patients (70.4%) received pegylated interferon α-2b (Peg-Intron, Schering-Plough) at a dosage of 1.5 μg/kg per week, and 61 patients (29.6%) received pegylated interferon α-2a (Pegasys, Roche), at a dosage of 180 μg per week. In patients infected by HCV genotypes 1, 4, and 5, ribavirin (either Rebetol, Schering-Plough, or Copegus, Roche) was administered according to body weight (1,000 mg/d for patients weighing <75 kg, 1200 mg/d for ≥75 kg); in the case of infection by genotypes 2 and 3, a flat ribavirin dose of 800 mg/d was used. The duration of therapy was 48 weeks for genotypes 1, 4, and 5 and 24 weeks for genotypes 2 and 3. The definitions of rapid viral response (RVR), complete early viral response (cEVR), end of treatment viral response (EOT), SVR, relapsers and nonresponders were made according to the American Association for the Study of Liver Diseases (AASLD) accepted guidelines.[16]

Table 2. Sustained Viral Response in Chronic HCV-Infected Patients Treated With PEG-Interferon and Ribavirin in Relationship to Baseline Demographic and Clinical Predictors

		All Genotypes			Genotypes 1/4-5		
		SVR+ N=131	SVR- N=75	P	SVR+ N=46	SVR- N=61	P
Age >50 years		58	32	0.823	14	24	0.340
Male gender		66	40	0.683	26	33	0.803
Body mass index >25		47	33	0.250	14	24	0.340
HOMA index	<2.0	69	36	0.514	25	30	0.586
	2.0–4.0	36	22	0.514	14	20	0.586
	>4.0	26	17		7	11	
Cholesterol ≤200 mg/dL		95	68	0.002	35	56	0.024
ALT >60 IU/mL		74	45	0.623	24	35	0.592
γGT >90 IU/mL		15	24	<0.001	7	20	0.038
HCV RNA >600.000 IU/mL		72	47	0.281	19	39	0.020
HCV genotype 1-4-5		46	61	<0.001	–	–	–
Use of PEG-interferon α-2b		88	57	0.182	26	46	0.039
Grading pretreatment >4*		17	12	0.492	4	9	0.201
Staging pretreatment >2*		35	26	0.148	16	22	0.411
Cumulative dose of IFN and RBV <80%		18	20	0.021	8	17	0.205
Alcohol consumption >20 g/day		16	8	0.739	6	7	0.806
IL-28B rs12979860 C/C		60	16	<0.001	24	8	<0.001

Data are presented for all and difficult to treat HCV genotypes. The statistical analysis was performed by means of Pearson chi-square test or chi-square test for linear trend when appropriate.

SVR, sustained viral response, HOMA, homeostasis model assessment, ALT, alanine aminotransferase, γGT, gamma-glutamyl transpeptidase, IFN, interferon α.

*Available in 142 patients.

Statistical Analysis. Statistical analysis of the data was performed using the BMDP dynamic statistical software package 7.0 (Statistical Solutions, Cork, Ireland). Continuous variables are presented as median (range) and categorical variables as frequencies (%). The associations between categorical variables were evaluated using the Pearson chi-squared test and, when appropriate, the chi-squared test for linear trend. The chi-squared G test "Goodness of Fit" was employed to verify whether the proportions of the genetic polymorphisms were distributed in accordance with the Hardy-Weinberg equation. Stepwise logistic regression analysis with a forward approach was performed to identify independent predictors of SVR and nonresponse.

Results

Viral Response. RVR was achieved by 103 patients (50.0%), cEVR by 150 (72.8%), EOT by 157 (76.2%), and SVR by 131 (63.6%) patients. The association between the achievement of SVR and the main clinical and demographic variables known to influence HCV viral clearance is reported in Table 2. In all patients, univariate analysis showed that the SVR rate was greatly influenced by viral genotype, IL-28B rs12979860 C/T polymorphism, baseline serum cholesterol, and gamma-glutamyltranspeptidase (γGT) levels. In difficult-to-treat genotypes, besides the prominent role exerted by IL-28B rs12979860 C/T polymorphism, the rate of SVR was also strongly associated with baseline serum HCV RNA and cholesterol levels.

25-OH Vitamin D Levels. The median value of circulating vitamin D was 20.7 (range 2.1-59.6) ng/mL. In all, 111 (53.9%) patients had normal (>20 ng/mL) 25-OH vitamin D serum levels, whereas the remaining 95 (46.1%) patients had vitamin D deficiency (≤20 ng/mL). Control subjects had a median vitamin D serum value of 28.4 (range 9.5-54.8) ng/mL. Low vitamin D levels (≤20 ng/mL) were detected in 95/206 patients with chronic hepatitis C and in 17/119 control subjects ($P < 0.0001$). Very low vitamin D levels (≤10 ng/mL) were detected in 32/206 patients with chronic hepatitis C and in 1/119 control subjects ($P < 0.0001$).

GC-Globulin Polymorphisms. In patients with chronic hepatitis C the following genotype frequencies were detected: rs7041 G>T polymorphism G/G = 64 (31.1%), G/T = 100 (48.5%), T/T = 42 (20.4%); rs4588 C>A polymorphism C/C = 108 (52.4%), C/A = 84 (40.8%), A/A = 14 (6.8%). The corresponding allele frequencies were: rs7041 G>T polymorphism G = 0.553 and T = 0.447; rs4588 C>A polymorphism C = 0.728 and A = 0.272. The genotype frequencies for both loci were not found to differ from what was expected from the Hardy-Weinberg equilibrium equation ($P > 0.5$ for both). The frequencies in controls were: rs7041 G>T polymorphism G/G = 40

Table 3. Association Between GC-Globulin Diplotypes (WT+/-) and Demographic and Clinical Variables in Chronic HCV-Infected Patients Treated With PEG-Interferon and Ribavirin

		All Genotypes			Genotypes 1/4-5		
		WT+ N=100	WT- N=106	P	WT+ N=53	WT- N=54	P
Age >50 years		44	46	0.930	19	19	0.943
Male gender		57	49	0.122	30	29	0.763
Body mass index >25		34	46	0.167	15	23	0.123
HOMA index	<2.0	56	49	0.225	33	22	
	2.0–4.0	25	33	0.225	14	20	0.025
	>4.0	19	24		6	12	
Cholesterol ≤200 mg/dL		80	83	0.764	46	45	0.616
ALT >60 IU/mL		50	69	0.028	24	35	0.042
γGT >90 IU/mL		11	28	0.005	7	20	0.005
HCV RNA >600.000 IU/mL		60	59	0.529	28	30	0.777
HCV genotype 1-4-5		53	54	0.768	–	–	–
25-OH vitamin D ng/mL	≤10	10	22		5	11	
	>10-≤40	79	78	0.017	40	42	0.009
	>40	11	6		8	1	
Grading pretreatment >4*		13	16	0.590	7	6	0.660
Staging pretreatment >2*		27	34	0.298	17	21	0.562
IL-28B rs12979860 C/C		42	34	0.140	19	13	0.184

The statistical analysis was performed by means of Pearson chi-square test or chi-square test for linear trend when appropriate.

GC-globulin diplotype WT+, carriage of at least three major alleles (G for the rs7041 and C for the rs4588), HOMA, homeostasis model assessment, ALT, alanine aminotransferase, γGT, gamma-glutamyl transpeptidase, IL-28B, interleukin 28B.

*Available in 142 patients.

(33.6%), G/T = 65 (54.6%), T/T = 14 (11.8%); rs4588 C>A polymorphism C/C = 59 (49.6%), C/A = 57 (47.9%), A/A = 3 (2.5%). The corresponding allele frequencies were: rs7041 G>T polymorphism G = 0.61 and T = 0.39; rs4588 C>A polymorphism C = 0.74 and A = 0.26. Adopting the dominant model for the major allele, patients were found to carry the minor allele of both loci more frequently than controls, although with a low level of significance (rs7041: 42/206 versus 14/119, $P = 0.047$; rs4588: 14/206 versus 3/119, $P = 0.095$). Haplotype frequencies in patients were: G-C = 55.1%, T-A = 26.9%, T-C = 17.7% and G-A = 0.3%. Diplotype frequencies were: G-C/G-C = 63 (30.5%), G-C/T-A = 64 (31.1%), G-C/T-C = 36 (17.5%), T-A/T-C = 19 (9.2%), T-A/T-A = 14 (6.8%), T-C/T-C = 9 (4.4%), G-C/G-A = 1 (0.5%). Table 3 illustrates the associations between clinical and demographic parameters and GC-globulin diplotypes. The carriage of the WT+ diplotype was associated in all patients with higher vitamin D levels and lower γGT serum values. The fact was confirmed in patients infected by HCV difficult-to-treat viral genotypes (Table 3).

GC-Globulin Polymorphisms, Vitamin D: Relationship With SVR and Nonresponse. Table 4 illustrates the rates of RVR, cEVR, EOT, and SVR as well as of nonresponse to antiviral treatment in relationship with the presence of normal vitamin D levels and with the carriage of the WT+ diplotype. A normal vitamin D value was mainly predictive of response in the initial steps of the treatment, whereas carrying the WT+ diplotype was important in determining later viral endpoints of the therapy. Interestingly, the carriage of the WT− diplotype was predictive of nonresponse to antiviral treatment. Combining vitamin D serum basal levels and GC-globulin polymorphism, four groups were identified: vitamin D ≤20 ng/mL and WT− (N = 51), vitamin D ≤20 ng/mL and WT+ (N = 44), vitamin D >20 ng/mL and WT− (N = 55), vitamin D >20 ng/mL and WT+ (N = 56). A significant linear trend was observed in the proportion of patients achieving SVR from the first to the last group: 29/51 (56.9%), 26/44 (59.1%), 33/55 (60.0%), 43/56 (76.8%, $P = 0.038$). An even higher linear stratification was detected in difficult-to-treat HCV genotypes: 6/25 (24.0%), 9/24 (37.5%), 12/29 (41.4%), 19/29 (65.5%, $P = 0.003$) (Fig. 1A).

GC-Globulin, Vitamin D, and IL-28B Polymorphisms: Relationship With SVR and Nonresponse. In patients carrying the IL-28B C/C genotype the rate of SVR achievement was not influenced by GC-globulin diplotypes. On the contrary, in patients carrying at least one IL-28B T allele, WT+ had higher rates of SVR in comparison to WT−. Thus, combining IL-28B and GC-globulin polymorphisms, SVR was found to increase starting from T/* plus WT− (33/72, 45.8%), to T/* plus WT+ (38/58, 65.5%), to C/C plus WT+/− (60/76, 78.9%) ($P < 0.0001$, Fig. 1B).

Table 4. Rates of Rapid, Complete Early, End of Treatment, Sustained Viral Response, Nonresponders, Relapsers, and Dropouts in Relationship With Normal Vitamin D Values and With GC-Globulin WT+ Diplotype

	All Genotypes					Genotypes 1/4-5				
	N=206					N=107				
	N	Vit. D >20 N=111	P	WT+ N=100	P	N	Vit. D >20 N=58	P	WT+ N=53	P
Rapid viral response	103	65 (58.6%)	0.008	51 (51.0%)	0.780	29	24 (41.4%)	<0.001	15 (28.3%)	0.782
Complete early viral response	150	89 (80.2%)	0.010	80 (80.0%)	0.024	56	38 (65.5%)	0.003	33 (62.3%)	0.042
End of treatment viral response	157	91 (82.0%)	0.036	85 (85.0%)	0.004	65	40 (69.0%)	0.058	40 (75.5%)	0.002
Sustained viral response	131	76 (68.5%)	0.116	69 (69.0%)	0.117	46	31 (53.4%)	0.017	28 (52.8%)	0.042
Nonresponders	32	14 (12.6%)	0.211	8 (8.0%)	0.004	32	14 (24.1%)	0.156	8 (15.1%)	<0.001
Relapsers	26	15 (13.5%)	0.677	16 (16.0%)	0.156	19	9 (15.5%)	0.510	12 (22.6%)	0.190
Dropout	17	5 (4.5%)	0.035	7 (7.0%)	0.526	11	4 (6.9%)	0.210	4 (7.5%)	0.356

Data are presented considering all patients and difficult to treat HCV genotypes.

Vit. D >20, basal Vitamin D serum levels >20 ng/mL. WT+, carriage of at least three major alleles (G for the rs7041 and C for the rs4588) for the GC-globulin.

The same occurred in difficult-to-treat HCV genotypes: T/* plus WT− (8/41, 17.4%), T/* plus WT+ (14/34, 30.4%), C/C plus WT+/− (24/32, 52.2%) ($P < 0.0001$, Fig. 1B). A strong stratification among groups was obtained simultaneously evaluating basal vitamin D levels and GC-globulin polymorphisms in relationship to IL-28B genotypes. SVR was attained in 14/35 (40.0%) IL-28B T/* genotypes and vitamin D ≤20 ng/mL and WT−, in 35/64 (54.7%) IL-28B T/* genotypes and either vitamin D levels ≤20 ng/mL or GC-globulin WT− diplotype, in 22/31 (71.0%) IL-28B T/* genotypes and vitamin D levels >20 ng/mL and GC-globulin WT+ diplotype, in 39/51 (76.5%) IL-28B rs12979860 C/C genotype and one or both vitamin D levels ≤20 ng/mL and GC-globulin WT− diplotype, in 21/25 (84.0%) IL-28B rs12979860 C/C genotype and vitamin D levels >20 ng/mL and GC-globulin WT+ diplotype ($P < 0.0001$). An even

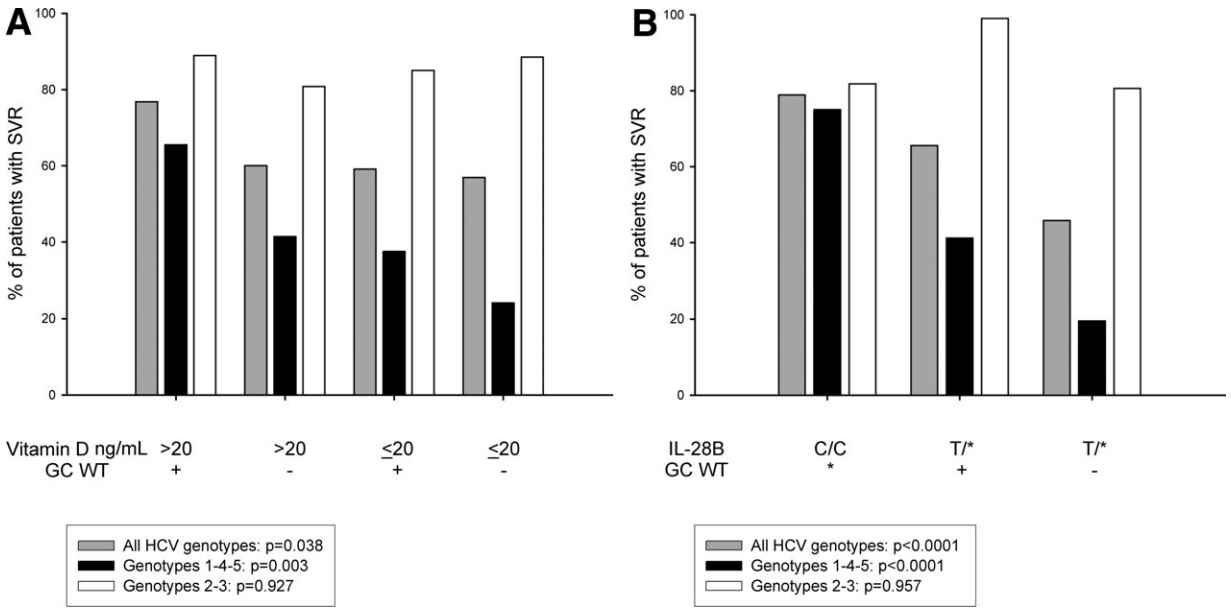

Fig. 1. (A) Rates of SVR in relationship with basal vitamin D serum levels (≤/>20 ng/mL) and GC-globulin rs7041 G>T and rs4588 C>A polymorphisms (WT+/−). Patients were divided into four groups: vitamin D levels >20 ng/mL and WT+ diplotype, vitamin D levels >20 ng/mL and WT− diplotype, vitamin D levels ≤20 ng/mL and WT+ diplotype, vitamin D levels ≤20 ng/mL and WT− diplotype. (B) Rates of SVR in relationship with the simultaneous assessment of IL-28B rs12979860 C/T polymorphism and GC-globulin rs7041 G>T and rs4588 C>A polymorphisms (WT+/−). Patients were divided into three groups: IL-28B rs12979860 C/C genotype irrespective of GC-globulin WT diplotype, IL-28B rs12979860 C/T or T/T genotypes and GC-globulin WT+ diplotype, IL-28B rs12979860 C/T or T/T genotypes and GC-globulin WT− diplotype. Results are presented in all HCV genotypes and separately in genotypes 1-4-5 and 2-3. The statistical analysis was performed by means of chi-square test for linear trend. WT+ diplotype: carriage of ≥3 major alleles (G-C/G-C, G-C/T-C, G-C/G-A), WT− diplotype: the remaining patients (G-C/T-A, T-A/T-C, T-A/T-A, T-C/T-C).

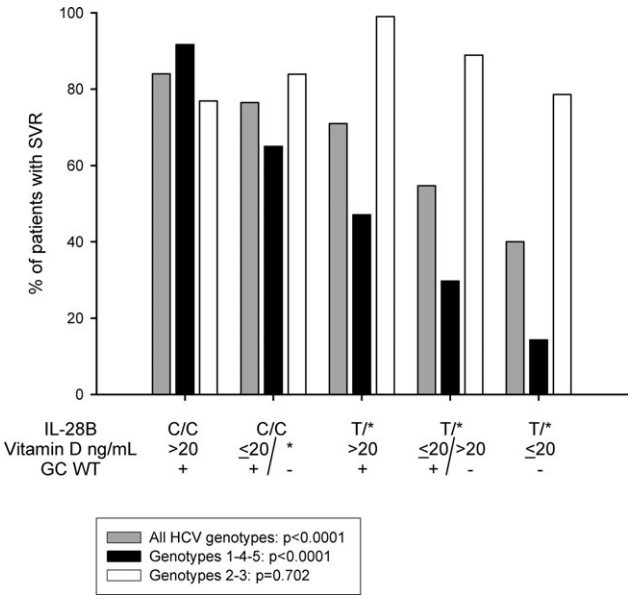

Fig. 2. Rates of SVR in relationship with IL-28B rs12979860 C/T polymorphism, vitamin D levels (≤/>20 ng/mL), and GC-globulin rs7041 G>T and rs4588 C>A polymorphisms (WT+/−). Patients were divided into five groups: IL-28B rs12979860 C/C genotype and vitamin D levels >20 ng/mL and GC-globulin WT+ diplotype, IL-28B rs12979860 C/C genotype and one or both vitamin D levels ≤20 ng/mL and GC-globulin WT− diplotype, IL-28B rs12979860 C/T or T/T genotypes and vitamin D levels >20 ng/mL and GC-globulin WT+ diplotype, IL-28B rs12979860 C/T or T/T genotypes and either vitamin D levels ≤20 ng/mL or GC-globulin WT− diplotype, IL-28B rs12979860 C/T or T/T genotypes and vitamin D levels ≤20 ng/mL and GC-globulin WT− diplotype. Results are presented in all HCV genotypes and separately in genotypes 1-4-5 and 2-3. The statistical analysis was performed by means of chi-square test for linear trend. WT+ diplotype: carriage of ≥3 major alleles (G-C/G-C, G-C/T-C, G-C/G-A), WT− diplotype: the remaining patients (G-C/T-A, T-A/T-C, T-A/T-A, T-C/T-C).

higher linear stratification was observed in difficult-to-treat HCV genotypes: 3/21 (14.3%), 11/37 (29.7%), 8/17 (47.1%), 13/20 (65.0%), 11/12 (91.7%) ($P < 0.0001$, Fig. 2).

Multivariate Analysis in the Prediction of SVR and Nonresponse. Table 5 illustrates the main demographic and clinical variables as well as IL-28B polymorphism in the prediction of viral nonresponse to interferon and ribavirin treatment in difficult-to-treat 1/4-5 HCV genotypes. Stepwise logistic regression analysis was performed in order to verify whether the interaction between GC-globulin polymorphisms and serum vitamin D could be considered an independent predictor of SVR. Variables included were those presented in Table 3 plus GC-globulin polymorphism diplotypes and their interaction with serum vitamin D. For the purposes of the analysis two groups were identified: patients with serum vitamin D >20 ng/mL and WT+ (N = 56) and the remaining patients (N = 150). Besides the major role played by HCV genotypes and IL-28B polymorphism, the interaction between serum vitamin D levels and GC-globulin diplotypes was found to be a strong independent predictor of SVR in HCV chronic infected patients treated with interferon α and ribavirin (Table 6). In difficult-to-treat HCV genotypes viral nonresponse was found to be independently associated with the carriage of the T allele of IL-28B polymorphism and with the carriage of the GC-globulin WT− diplotypes (Table 6).

Discussion

GC-globulin is a serum glycosylated α_2-globulin with a molecular weight of about 58 kDa. The amino acid sequence is composed of 458 residues arranged in three domains. Low serum levels have been detected in critically ill patients with sepsis or organ dysfunction and in the presence of more advanced liver fibrotic stages.[17] GC-globulin gene is localized on the long arm of chromosome 4 and belongs to a cluster composed of four genes including albumin, α-fetoprotein and α-albumin/afamin. The marked homology in nucleotides and amino acid sequences among these proteins supports the hypothesis that these four genes derive from a unique ancestral gene.[18] All these four

Table 5. Viral Nonresponse in Chronic HCV-Infected Patients Treated with PEG-Interferon and Ribavirin in Relationship to Baseline Demographic and Clinical Predictors

		Genotypes 1/4-5		
		Nonresponse N=32	Response N=75	P
Age >50 years		12 (37.5%)	26 (34.7%)	0.779
Male gender		15 (46.9%)	44 (58.7%)	0.262
Body mass index >25		12 (37.5%)	26 (34.7%)	0.779
HOMA index	<2.0	14 (43.4%)	41 (54.7%)	
	2.0–4.0	11 (34.4%)	23 (30.7%)	0.252
	>4.0	7 (21.9%)	11 (14.7%)	
Cholesterol ≤200 mg/dL		29 (90.6%)	62 (82.7%)	0.291
ALT >60 IU/mL		19 (59.4%)	40 (53.3%)	0.565
γGT >90 IU/mL		15 (46.9%)	12 (16.0%)	<0.001
HCV RNA >600.000 IU/mL		19 (59.4%)	39 (52.0%)	0.483
Use of PEG-interferon α-2b		23 (71.9%)	49 (65.3%)	0.509
Grading pretreatment >4*		3 (12.0%)	10 (16.7%)	0.586
Staging pretreatment >2*		12 (48.0%)	26 (43.3)	0.693
Cumulative dose of IFN and RBV <80%		7 (21.9%)	18 (24.0%)	0.812
Alcohol consumption >20 g/day		4 (12.5%)	9 (12.0%)	0.942
IL-28B rs12979860 C/C		2 (6.2%)	30 (40.0%)	<0.001

Data are presented for 1/4-5 HCV genotypes; in easy to treat 2-3 HCV genotypes no nonresponse was detected. The statistical analysis was performed by means of Pearson chi-square test or chi-square test for linear trend when appropriate.

HOMA, homeostasis model assessment, ALT, alanine aminotransferase, γGT, gamma-glutamyl transpeptidase, IFN, interferon α.

*Available in 142 patients.

Table 6. Summary of the Results of Stepwise Logistic Regression Analysis in the Prediction of Sustained Viral Response in All HCV Genotypes

	Sustained Viral Response						Nonresponse		
	All HCV Genotypes			HCV Genotypes 1/4-5			HCV Genotypes 1/4-5		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
HCV genotypes 2-3	9.58	4.47-20.5	<0.001	–	–	–	–	–	–
IL-28B C/C genotype	3.25	1.49-7.05	0.003	12.5	3.77-41.7	<0.001	0.090	0.018-0.452	<0.001
Vitamin D >20 ng/mL / WT+	3.30	1.44-7.55	0.015	4.52	1.47-13.9	0.015	–	–	–
HCV RNA ≤600.000 I.U./mL	2.53	1.21-5.26	0.010	5.77	1.96-17.0	0.001	–	–	–
Serum cholesterol mg/dL	1.01	1.00-1.02	0.008	1.02	1.00-1.04	0.010	0.981	0.965-0.998	0.019
GC-globulin diplotypes WT+	–	–	–	–	–	–	0.164	0.056-0.482	0.002

In difficult to treat 1/4-5 HCV genotypes results concerning both sustained viral response and no-response are reported. The variables included in the analysis were those reported in table 2 (except for histology available in 142 patients), GC-globulin diplotypes (WT +/-), serum vitamin D (≤/>20 ng/mL) and their interaction: vitamin D >20 ng/mL plus GC WT+ (N=56) versus the remaining patients (N=150).

OR, odds ratio, CI, confidence interval, IL-28B, interleukin 28B, GC-globulin diplotype WT+, carriage of at least three major alleles (G for the rs7041 and C for the rs4588) for the GC-globulin.

genes are expressed almost exclusively in the liver. A considerable GC-globulin polymorphism has been demonstrated in humans and primates initially using electrophoretic methods.[19] Three predominant isoforms have been described, originating from the genetic recombination of the two main polymorphic loci: the rs7041 G>T and the rs4588 C>A transversions. The isoform 1f encompasses the rs7041 T and rs4588 C haplotype, the 1s the G-C haplotype, and finally the isoform 2 encompasses the T-A haplotype.

In this study, as already reported in the literature concerning Caucasian populations,[20] the G allele of the rs7041 G>T and the C allele of the rs4588 C>A polymorphisms of GC-globulin gene were found to be the major alleles. Taking particularly into account haplotype frequencies, GC-1s was the more frequent form found in comparison to GC-1f, whereas GC-2 was far less frequent; in our series the very rare GC G-A haplotype was present only in one patient. The primary amino acid sequences of GC-1s and GC-1f isoforms are identical, except for the 416 position where glutamic acid is substituted by aspartic acid. On the other hand, GC-1f and GC-2 differ by a single amino acid modification in position 420 (threonine versus lysine). These variations were associated with lower GC-globulin circulating levels.[21]

Several studies have been performed to ascertain whether GC-globulin polymorphisms influence serum vitamin D levels.[14] There is substantial agreement that the carriage of the major alleles of GC rs7041 G>T and rs4588 C>A polymorphisms, in particular the GC-1s and GC-1f isoforms, is associated with higher serum vitamin D. To assess the overall influence of the two polymorphic loci of GC-globulin on vitamin D levels, diplotype analysis was performed. Patients were divided into two groups: the first (GC-globulin WT+) composed of those carrying 3 or 4 major alleles of rs7041 G>T and rs4588 C>A polymorphic loci, the second (GC-globulin WT−) composed of the remaining patients. A significant association was detected between the carriage of WT− diplotype and the presence of lower vitamin D values.[14] Although patients were found to carry the minor allele of both loci more frequently than controls, no difference was observed in diplotype frequencies between the two groups. On the contrary, vitamin D values were found to be exceedingly lower in patients with chronic hepatitis C in comparison to matched control subjects, as reported.[22] The degree of fibrosis in chronic liver diseases has been found to be a strong determinant of vitamin D deficiency, due to the implication of liver function in vitamin D metabolism.[9] Nevertheless, no clear explanation exists about the etiological role of HCV in determining, even in the presence of modest liver fibrosis, a fall in vitamin D levels. A direct implication of HCV in interfering in the cellular pathways of vitamin D activation has been supposed. Although whether vitamin D deficiency associates or not with faster fibrosis progression in chronic hepatitis C is debated.[23,24]

In this study the rate of SVR in all patients was found to be 63.6%; in agreement with that reported in the literature, it was 43.0% in difficult-to-treat and 85.9% in easy-to-treat HCV genotypes.[25] IL-28B rs12979860 C/T polymorphism was confirmed to play a pivotal role in predicting the rate of SVR,[7] independently of other well-known predictors, such as HCV genotype, HCV viral load, and baseline serum cholesterol. The novelty of this study resides in the fact that for the first time vitamin D axis determinants were found to greatly influence the rate of viral

clearance after interferon and ribavirin treatment in patients with chronic HCV infection. Although serum basal vitamin D deficiency seems to greatly affect the initial processes leading to viral decline, genetic determinants regulating vitamin D appear to possess a persistent influence on vitamin D-related immunological mechanisms associated with HCV clearance. Accordingly, a strong association was found between the interaction between vitamin D levels (≤/>20 ng/mL) and GC-globulin diplotype carriage (WT+/WT−) and HCV clearance in difficult-to-treat genotypes. Patients possessing normal vitamin D levels and carrying the WT+ diplotype achieved SVR in 65.5% of the cases; at the opposite, those with vitamin D deficiency and carrying the WT− diplotype achieved SVR only in 24.0% of the cases.

Although an extensive analysis has been previously performed on predictors of SVR achievement in patients with chronic hepatitis C, scarce data are present in the literature on the prediction of nonresponse to antiviral treatment.[4] In the present study about one-third of patients infected by difficult-to-treat HCV genotypes were found to be nonresponders to interferon plus ribavirin therapy. This result is similar to that previously detected in a larger series.[25] Carriage of the T allele of IL-28B rs12979860 C/T polymorphism was confirmed to be associated with nonresponse.[26] Interestingly, in this study, for the first time the carriage of the GC-globulin WT− diplotype was strongly and independently associated with the condition of nonresponder to antiviral therapy. Thus, it may be postulated that the capacity of induce a positive response to antiviral therapy in chronic HCV infection could be influenced by the presence of an efficient vitamin D transport capacity in turn conditioning a normal vitamin D bioavailability. In fact, vitamin D has been suggested to possess an antiviral effect in an *in vitro* infectious HCV production system.[27] Low vitamin D levels were associated *in vivo* with a poor response to antiviral treatment for patients affected by chronic hepatitis C.[9,10,28]

Besides its role as vitamin D transporter, GC-globulin possesses a number of other functions implicated in the modulation of the inflammatory response. First, GC-globulin has the ability to bind actin released into the circulation by necrotic cell destruction, thus protecting against disseminated intravascular coagulation.[29] Second, GC-globulin may be converted, following partial deglycosylation operated by enzymes released by inflammatory cells, into the GC-macrophage activating factor (GC-MAF).[30] Finally, GC-globulin can transport lipids such as arachidonic acid and endotoxin and can exert immunomodulatory function enhancing the chemotactic activity of the C5a.[31] Altogether, these biological functions could imply a vitamin D-independent role of GC-globulin polymorphisms in influencing the response to interferon in the treatment of hepatitis C.

In patients infected by difficult-to-treat HCV genotypes, may vitamin D axis assessment improve pretreatment prediction of antiviral standard therapy outcome? Looking at the probability of SVR achievement, one might first of all demonstrate that GC-globulin evaluation could improve the power of the major host genetic predictor, IL-28B rs12979860 C/T polymorphism. In effect, carriage of the IL-28B C/C genotype was confirmed to possess great strength in the prediction of a positive outcome of therapy. Interestingly, in patients carrying the less favorable IL-28B rs12979860 C/T and T/T genotypes, carriage of the WT+ diplotype was associated with a doubling chance of SVR in comparison to the carriage of WT− GC-globulin diplotype (41.2% versus 19.5%). Finally, GC-globulin polymorphisms evaluation appears to be interesting in the identification of true nonresponders to antiviral treatment and therefore in predicting patients prone to have interferon resistance. In this category of patients, interferon-based antiviral treatment, although clinically indicated, would be associated with a poor response rate not modifiable by adding the new direct antiviral agents.

This study has some limitations. First, it analyzed only Caucasian subjects of northern Italy; thus, the results could be replicated in other ethnicities carrying different allelic frequencies for the GC-globulin rs7041 and rs4588 loci. Second, the design of the study is retrospective. Third, only a baseline serum vitamin D determination was available and analyzed; moreover, no GC-globulin serum levels were tested because stored serum was available only in a minority of patients.

In conclusion, genetic study of IL28B could be integrated with the genetic study of GC-globulin polymorphisms to better identify pretreatment patients with a higher probability to have interferon resistance, a condition limiting the successful use of new direct antiviral agents. In difficult-to-treat HCV genotypes, simultaneous pretreatment normal serum vitamin D levels and carriage of the GC-globulin wildtype isoform strongly predict the achievement of SVR after PEG-interferon plus ribavirin antiviral therapy.

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