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Original Article

IL-28B genotypes as predictors of long-term outcome in patients with hepatitis C-related severe liver injury

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Abstract

Introduction: Patients with severe fibrosis or cirrhosis are at high risk for liver-related complications, even after successful antiviral treatment and/or regression of fibrosis. These are the first published results concerning the role of *IL-28B* genotypes as predictors of the durability of sustained virological response (SVR) and long-term outcome, in patients with baseline severe fibrosis and cirrhosis caused by hepatitis C (HCV) infection.

Methodology: Genetic testing for three different single nucleotide polymorphisms (SNP) near the *IL28B* gene, rs12979860, rs12980275 and rs8099917, was performed in 42 patients with HCV-related advanced fibrosis and cirrhosis, who achieved SVR after successful interferon-based treatment. Baseline clinical and laboratory parameters were analysed, as well as *IL28B* genotype association with late virological relapse, fibrosis progression and clinical outcomes.

Results: The most prevalent genotypes in all three tested SNP positions were: $CC_{rs12979860}$ genotype in 69% of patients, $GT_{rs8099917}$ in 78.6% and $GG_{rs12980275}$ in 47.6% of patients. The presence of *IL28B* $CC_{rs12979860}$ genotype was identified as a negative predictor of late virological relapse. Further analysis did not confirm the association of other *IL28B* genotypes with the progression of fibrosis and clinical outcomes.

Conclusions: Varying long-term prognosis in patients with HCV-related severe fibrosis and cirrhosis is due to multiple interactions between host genetic factors, virus and environment. These are first published results demonstrating the significance of *IL28B* $CC_{rs12979860}$ genotype as a negative predictor of late virological relapse. A further investigation concerning genetic factors is necessary to identify patients under risk for late relapse, complications and unfavorable outcomes, so that they can be reevaluated and offered new treatment options.

Key words: interleukine 28B; late relapse; outcome.

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Introduction

Long-term outcome of chronic hepatitis C (CHC) is variable in both treatment-naive and treatment-experienced patients and influenced by numerous virus-related, environmental and host factors [1]. So far, many genetic markers have been taken in account and examined as possible predictors of outcome, including several single-nucleotide polymorphisms (SNPs) located in various genes, such as interleukine 28B region on chromosome 19, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) [2]. Genome Wide Association Studies (GWAS) have shown that multiple SNPs located near IL28B gene are responsible for the occurrence of different *IL28B* genotypes and have a significant impact on spontaneous and treatment clearance of hepatitis C virus (HCV) [3,4]. Unfavourable treatment outcomes have been documented in patients with the TT and CT genotypes at the rs12979860 SNP (nonresponder genotypes), including delayed viral decline and lower treatment success rates, after pegylated interferon and ribavirin treatment, contrary to the favourable CC genotype [5]. The impact of *IL28B* on the natural history of CHC infection is still not well understood [6]. Furthermore, there are conflicting results concerning the predictive role of *IL28B* genotypes in fibrosis progression, including *IL28B* $CC_{rs12979860}$ genotype association with

higher levels of ALT, adverse clinical outcomes and greater hepatic necroinflammation [6].

Estimated seroprevalence of HCV infection in Serbia is higher than in most of the developed European countries, approximately 0.19%, with the predominance of 1b and 3a genotypes [7,8]. Although antiviral treatment (pegylated interferon and ribavirin) has been available since 2003. with high treatment success rates, decades of treatment experience and close follow-up have demonstrated, in our patient population, that liver-related morbidity and mortality is still high in patients with a severe baseline liver damage, in spite of achieving sustained virologic response (SVR). Apart from routine clinical and laboratory outcome predictors, we often suspect that there is a significant impact of host genetic factors on the clinical course and durability of SVR, especially in relation to the presence of IL-28B genotype. In Serbia, there are limited data concerning the role of *IL-28B* genotypes as treatment response predictors in patients with CHC (only in HCV genotype 1 infection), but with no long-term follow up studies or published data beyond achieving SVR [9].

The aim of this study was to examine if *IL-28B* genotypes can predict the occurrence of late virological relapse, the progression of fibrosis and to examine its' association with the long-term outcome in Serbian cohort of patients with CHC-related severe baseline liver damage, who achieved SVR after interferon-based treatment. Host and virus-related factors were also included in the analysis as possible predictors of the durability of SVR and long-term outcome, as well as their association with different *IL-28B* genotypes.

Methodology

This prospective cohort study was conducted in the referral centre at the Hepatology Department of the Clinic for Infectious and Tropical Diseases, Medical Faculty, University of Belgrade, Clinical Center of Serbia. It included a study group of 42 patients with severe baseline liver damage caused by CHC, in whom IL-28B genotyping was available and who had fulfilled all inclusion criteria. These patients were a part of a larger long-term follow-up study of 325 patients with CHC, treated/retreated with pegylated interferon and ribavirin during a 12 years' period (from 2003 until November 2015). All patients from this large cohort, who had successfully undergone antiviral treatment and achieved SVR were contacted for a reevaluation during March-July 2018. This included physical exam, liver elastography, HCV RNA loads, liver and genomic biomarkers. Among subjects who responded, 42 patients fulfilled the inclusion criteria and consented to participate in this study. Two patients from this group had been retreated during this period and achieved SVR after retreatment, so they were also recruited. The pre-treatment data were obtained from patient files, including demographic and epidemiological data, baseline clinical findings, laboratory tests and liver histology (before the commencement of antiviral treatment). Regular outpatient monitoring included clinical examinations every 6 months, laboratory tests (haematological, biochemical, HCV RNA loads, HCV genotyping in late relapse), imaging (fibroscan and echosonography yearly) and upper gastrointestinal endoscopy (in patients with cirrhosis). Among these 42 patients, 9 had been tested for HCV RNA prior to our reevaluation during follow-up and already had a confirmed virological relapse, in spite of achieving SVR.

The inclusion criteria for the study group were: patients with a detectable HCV RNA by polymerase chain reaction (PCR) and advanced fibrosis or cirrhosis (METAVIR score F3 and F4), who underwent a complete treatment with pegylated interferon and ribavirin, achieved SVR (e.g. undetectable HCV RNA 6 months after the completion of antiviral treatment) and gave informed consent for participation. The exclusion criteria for study group were: age (subjects younger than 16 or older than 65 years), mild liver damage with METAVIR < 2 confirmed by histopathology, coinfections (hepatitis B, HIV), other liver diseases (Wilson's disease, haemochromatosis, autoimmune hepatitis, primary biliary cirrhosis, baseline decompensated cirrhosis and hepatocellular carcinoma-HCC), severe comorbidities (decompensated cardiomyopathies, thyroid dysfunctions, autoimmune diseases, epilepsy, depression, severe neutropenia and thrombocytopenia), active abuse of narcotics and alcohol, pregnancy. The study protocol was designed according to the ethical guidelines of the Helsinki declaration and was approved by the Ethical Committee of Medical Faculty, University of Belgrade.

Baseline liver damage assessment included non-invasive (liver elastography) and invasive (aspiration liver biopsy, Institute for Pathology, Medical Faculty Belgrade University) diagnostic methods, and METAVIR score was used to quantify the degree of inflammation and fibrosis of the liver. Viral loads were measured with quantitative PCR HCV RNA (Cobas Amplicor HCV Test version 2.0, Roche Diagnostics, Menheim, detection: 50 IU/mL) and hepatitis C virus genotyping (Linear Array HCV genotyping test, Roche Diagnostics, Mannheim, Germany) in Virology

laboratory, Microbiology Department, Clinical Center of Serbia. Biochemical testing was performed using Siemens Dimension Xpand biochemistry analyzer in the Centre for Medical Biochemistry, Department of the Clinic for Infectious and Tropical Diseases, Clinical Centre of Serbia.

Study endpoints during follow-up period included late virological relapse, increase of alanine aminotransferase (ALT) levels and unfavourable clinical outcomes. Late relapse was considered in patients who had detectable HCV RNA during follow up, in spite of achieving SVR, and in whom repeated virus genotyping matched initial pretreatment HCV genotype. The progress of fibrosis was assessed using repeated non-invasive liver elastography. Clinical outcomes, which were taken into consideration, included cirrhosis decompensation, ascites, variceal bleeding, spontaneous bacterial peritonitis, hepatic encephalopathy and hepatocellular carcinoma (HCC).

All analyzed subjects were Caucasians of Serbian origin. Molecular genetic analysis was performed at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade.

Genomic DNA was extracted from peripheral blood samples of the patients using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The detection of three SNPs near the *IL28B* gene, rs12979860, rs12980275 and rs8099917, was performed using sequence specific primer – polymerase chain reaction (SSP-PCR), as previously described [10].

Statistical analysis was performed using SPSS® v. 17 and included both descriptive and analytical methods. Data were presented as a percent or mean value with SD. Pretreatment data were analyzed using parametric (Student T-test, ANOVA) and nonparametric tests (Mann-Whitney, χ^2, Kruskal Wallis), depending on the normality of variables. Further genotype associations with different endpoints were described using relative risks, and tested with logistic regression and beta coefficient, including Cox regression for a time-dependent association. Values at the $p \leq 0.05$ level were considered statistically significant, the confidence interval (CI) was 95% and all tests were 2-tailed.

Results

The study group included a total of 42 patients who had achieved SVR, 46.2 ± 9.4 years old (ranging from 27 up to 63), including 24 (57.1%) males, with a variable pretreatment duration of infection ranging from 6 months to 13 years. The predominant route of infection in 40.5% of patients was blood transfusion (P = 0.005).

Table 1. Comparison of baseline (pretreatment) clinical, laboratory and histology findings in patients with three rs12979860 polymorphism genotypes (CC, CT and TT), including a comparison of patients with $CC_{rs12979860}$ genotype versus non-CC genotype.

Variable	CC	CT	TT	P	CC *vs.* non CC
patients, n (%)	29 (69%)	7 (16.7%)	6 (14.3%)	*< 0.0001*	*0.014*
male sex, n (%)	17 (58.6%)	4 (57.1%)	3 (50%)	0.077	0.773
age, years*	47±9.5	39.4 ± 7.69	50.3 ± 7	0.07	0.418
duration of infection, years*	3.4 ± 4.1	2 ± 1.6	3 ± 4.4	0.704	0.469
BMI*	21.45 ± 1.9	21.5 ± 1.6	21.2 ± 1.7	0.969	0.926
cirrhosis (METAVIR F4), n (%)	7 (24.1%)	3 (42.9%)	4 (66.7%)	0.445	*0.05*
high activity, n (%)	7 (24.1%)	5 (71.4%)	4 (66.7%)	*0.02*	*0.029*
HCV genotype 1, n (%)	21 (72.4%)	3 (42.9)	4 (66.7%)	0.581	0.501
logHCV RNA IU/mL *	5.95 ± 0.65	6.1 ± 0.7	6.56 ± 0.5	0.12	0.124
HCV RNA > 800.000 IU/mL, n (%)	18/29 (62.1%)	5/7 (71.4%)	5/6 (83.3%)	0.578	*0.014*
Hgb (g/L)*	138 ± 18	153 ± 6.9	151.2 ± 9.5	0.249	0.135
RBC ($\times 10^6$ cells/μL)*	4.5 ± 0.63	4.5 ± 0.59	4.8 ± 0.5	0.286	0.16
WBC ($\times 10^3$ cells/μL)*	7 ± 1.8	6.9 ± 0.56	8.95 ± 2.96	*0.039*	0.36
Platelets ($\times 10^3$ cells/μL)*	192.5 ± 70	185 ± 33.9	222 ± 86.6	0.515	0.633
AST (U/L)*	65.7 ± 47.7	64.2 ± 19.5	80.83 ± 52.6	0.702	0.639
ALT (U/L)*	132.46 ± 128.7	110.4 ± 47.9	157 ± 151	0.783	0.882
GGT (U/L)*	61.5 ± 64.5	81.6 ± 39.1	78.2 ± 72	0.55	0.28
total bilirubin (μmol/L)*	12.7 ± 6.8	12.5 ± 6.5	14.45 ± 6.11	0.374	0.869
ferritin (μg/mL)*	155.9 ± 144.3	209.6 ± 136	68.5 ± 31.65	0.227	0.748
total proteins (g/L)*	72.7 ± 7	75 ± 7	66.8 ± 14	0.199	0.658
albumin (g/L)*	39.2 ± 8.6	44.7 ± 5.8	39.5 ± 6.8	0.272	0.257

BMI- the body mass index; Hgb-hemoglobin; RBC-red blood cell count; WBC-white blood cell count; AST-aspartate aminotransferase; ALT-alanine aminotransferase; GGT- gamma-glutamyl transferase;*mean ± SD.

Table 2. Comparison of baseline (pretreatment) clinical, laboratory and histology findings in patients with three rs8099917 polymorphism genotypes (GT, GG and TT), including a comparison of patients with $TT_{rs8099917}$ genotype versus non-TT genotype.

Variable	G/T	G/G	T/T	P	TT *vs.* non TT
patients, n (%)	33 (78.6%)	5 (11.9%)	4 (9.5%)	*0.000*	*0.000*
male sex, n (%)	17 (51.5%)	3 (60%)	4 (100%)	0.179	0.070
age, years*	46.1 ± 9.3	47.6 ± 13	45.5 ± 6.1	0.938	0.871
duration of infection, years*	3.4 ± 4	2.5 ± 3.1	1.1 ± 0.7	0.584	0.359
BMI*	20.9 ± 1.7	23.3 ± 1.1	23 ± 0.8	*0.003*	0.069
cirrhosis (METAVIR F4), n (%)	12 (36.4%)	2 (40%)	0 (0%)	0.327	0.137
high activity, n (%)	13 (39.4%)	1 (20%)	2 (50%)	0.544	0.617
HCV genotype 1, n (%)	21 (63.6%)	3 (60%)	4 (100%)	0.466	0.331
logHCV RNA IU/mL*	6.03 ± 0.64	5.98 ± 0.78	6.33 ± 0.8	0.376	0.394
HCV RNA > 800.000 IU/mL, n (%)	20 (60.6%)	4 (80%)	4 (100%)	0.229	0.283
Hgb (g/L)*	141.8 ± 16.4	143.6 ± 21.3	143 ± 17.4	0.931	0.847
RBC ($\times 10^6$ cells/μL)*	4.5 ± 0.6	4.5 ± 0.7	4.5 ± 0.5	0.972	0.820
WBC ($\times 10^3$ cells/μL)*	6.9 ± 1.9	7.6 ± 1.3	7.47 ± 3.6	0.741	0.688
Platelets ($\times 10^3$ cells/μL)*	192.5 ± 69	196.6 ± 35	208 ± 96	0.886	0.645
AST (U/L)*	62.6 ± 32.8	76 ± 79.4	88.5 ± 69	0.479	0.297
ALT (U/L)*	116 ± 77	164 ± 248	170 ± 193	0.547	0.455
GGT (U/L)*	60.8 ± 61	101 ± 64	53.5 ± 15.2	0.390	0.643
total bilirubin (μmol/L)*	12.2 ± 6.2	15.8 ± 9.35	11.7 ± 2.8	0.482	0.772
ferritin (μg/mL)*	162.5 ± 138.5	114 ± 125.6	91.25 ± 92	0.551	0.390
total proteins (g/L)*	72.68 ± 9.3	71.8 ± 9.2	70.5 ± 2.4	0.900	0.671
albumin (g/L)*	41.5 ± 7.9	35.6 ± 8	35.3 ± 9.2	0.143	0.206

BMI- the body mass index; Hgb-hemoglobin; RBC-red blood cell count; WBC-white blood cell count; AST-aspartate aminotransferase; ALT-alanine aminotransferase; GGT- gamma-glutamyl transferase; *mean ± SD.

Table 3. Comparison of baseline (pretreatment) clinical, laboratory and histology findings in patients with three rs12980275 polymorphism genotypes (GG, AG and AA), including a comparison of patients with $AA_{rs12980275}$ genotype versus non-AA genotype.

Variable	G/G	A/G	A/A	P	AA *vs.* non AA
patients, n (%)	20 (47.6%)	16 (38.1%)	6 (14.3%)	*0.024*	*0.000*
male sex, n (%)	12 (60%)	8 (50%)	4 (66.7%)	0.733	0.685
age, years*	47 ± 9.4	46.7 ± 10	43.6 ± 9	0.777	0.475
duration of infection, years*	2.4 ± 2.9	4.2 ± 4.6	2.8 ± 4	0.400	0.832
BMI*	21.7 ± 1.8	20.8 ± 1.8	21.8 ± 1.9	0.280	0.616
cirrhosis (METAVIR F4), n (%)	7 (35%)	6 (37.5%)	1 (16.7%)	0.638	0.096
high activity, n (%)	9 (45%)	4 (25%)	3 (50%)	0.381	*0.021*
HCV genotype 1, n (%)	14 (70%)	12 (75%)	2 (33.3%)	0.440	0.168
log HCV RNA IU/mL*	6.1 ± 0.4	6.07 ± 0.7	5.8 ± 1	0.748	0.450
HCV RNA > 800.000IU/mL, n (%)	15 (75%)	11 (68.8%)	2 (33.3%)	0.161	*0.000*
Hgb (g/L)*	138.5 ± 19.7	142.7 ± 13.8	147.33 ± 15.6	0.512	0.364
RBC ($\times 10^6$ cells/μL)*	4.48 ± 0.7	4.5 ± 0.5	4.86 ± 0.5	0.419	0.188
WBC ($\times 10^3$ cells/μL)*	7 ± 2.2	6.96 ± 1.3	7.4 ± 3.2	0.897	0.654
Platelets ($\times 10^3$ cells/μL)*	207.9 ± 70	162.75 ± 54.2	207.95 ± 70	0.060	0.227
AST (U/L)*	50 ± 20	78 ± 48	89.6 ± 68.35	0.060	0.164
ALT (U/L)*	81.4 ± 42.9	143.8 ± 147.5	228.5 ± 139.6	*0.022*	*0.021*
GGT (U/L)*	81.42 ± 75.3	51.75 ± 42.8	48.5 ± 17.3	0.191	0.422
total bilirubin (μmol/L)*	12.7 ± 6.6	12.6 ± 7.2	12.1 ± 3.3	0.978	0.845
ferritin (μg/mL)*	176.8 ± 146	140 ± 99	87.8 ± 164	0.427	0.249
total proteins (g/L)*	71.1 ± 11.5	73.25 ± 5.5	74 ± 6	0.659	0.608
albumin (g/L)*	40.8 ± 7.6	39.37 ± 9.6	40.2 ± 6.6	0.877	1.000

BMI- the body mass index; Hgb-hemoglobin; RBC-red blood cell count; WBC-white blood cell count; AST-aspartate aminotransferase; ALT-alanine aminotransferase; GGT- gamma-glutamyl transferase; *mean ± SD.

Baseline demographic, clinical and laboratory findings

Baseline analysis of rs12979860 polymorphism genotypes frequencies revealed the predominance of $CC_{rs12979860}$ genotype in 29/42 (69%) patients, compared to other less favourable genotypes CT (16.7%) and TT (14.3%) ($P < 0.0001$). There were no significant host-related differences (such as sex distribution, age, duration of infection, BMI) between patients with different genotypes (Table 1). However, in patients with non-CC genotypes compared to patients with $CC_{rs12979860}$ genotype, higher histological activity of hepatitis (69.2% *vs*. 24.1%) and cirrhosis (53.8% *vs*. 24.1%) were more prevalent ($P < 0.05$). High baseline viraemia was noted in patients with non-CC genotype, with more patients having HCV RNA loads above 800.000 IU/mL compared to those with CC genotype ($P = 0.014$). Patients with TT genotype had higher baseline white blood cell (WBC) counts compared to CT and CC genotypes (8.95 ± 2.9 *vs* 6.9 ± 0.56 *vs*. 7 ± 1.8, $P = 0.039$), but there was no statistically significant difference between patients with CC and non-CC genotypes ($P = 0.360$). There were no significant differences in average values of other haematological and biochemical findings between patients with CC and non-CC genotypes (Table 1).

Further baseline analysis of rs8099917 polymorphism genotype frequency (Table 2) revealed a predominance of $GT_{rs8099917}$ genotype in 33/42 patients (78.6%) ($P < 0.0001$). The only significant differences among three groups of patients, according to their genotype, was in average BMI, but there were no significant differences between TT and non-TT genotypes.

Study group baseline analysis of rs12980275 polymorphism (Table 3) showed a predominance of $GG_{rs12980275}$ genotype in 20/42 patients (47.6%, $P = 0.02$). A more favourable $AA_{rs12980275}$ genotype found in 6/42 of patients (14.3%) was the most infrequent, compared to non-AA genotypes ($P < 0.000$). Furthermore, patients with AA genotype had a higher baseline histological activity of hepatitis compared to non-AA genotypes (50% of patients had high activity *vs*. 36.1%, $P = 0.021$), and higher pretreatment activity of ALT ($P = 0.022$).

Association of IL28B genotypes with virological relapse, increase in ALT levels, progression of fibrosis and unfavourable clinical outcomes

The follow-up period of patients, after achieving SVR, ranged from 20 months to 11.3 years (66.9 ± 37.5 months), but during the last follow-up visit, HCV RNA testing confirmed detectable viremia in 14 patients (33.3%), who were considered late relapsers. Non-CC genotype was more common in patients with late relapse, in 8/14 (57.1%) patients compared to 6/14 (42.9%) of patients with $CC_{rs12979860}$ genotype ($P = 0.015$), with 6 times greater relative risks for virological failure (RR = 3.2; 95%CI 1.278-7.982 *vs*. RR = 0.522; 95%CI 0.278-0.979) respectively. Further analysis (Table 4) supported by logistic regression revealed a significant negative association of $CC_{rs12979860}$ genotype with late virological relapse ($\beta = -1.814$, $P = 0.013$). Genotype $TT_{rs12979860}$ presence had the highest relative risk for late relapse (RR = 2.000, 95% CI 0.462-8.664), but no statistically significant association was established including other three examined genotypes (Table 4).

The elevation of ALT was observed in 10.3% of patients with $CC_{rs12979860}$ genotype, and in 15.4% with non-CC genotype. Although the difference in frequencies between these two groups did not reach significance level ($P = 0.637$), relative risks for the ALT elevation (independent of viraemia), were 1.5 times greater in non-CC genotype *vs*. CC genotype patients (RR = 1.345, CI 95% 0.412-4.389 *vs*. RR = 0.854 CI 95% 0.405-1.800). Cox regression did not reveal a time-dependent association between $CC_{rs12979860}$ genotype and elevation of ALT ($\beta = -0.353$ $P = 0.723$).

Among patients with $CC_{rs12979860}$ genotype estimated progression of fibrosis was observed in 6/29 (20.7%) patients, similar to patients with non-CC genotype 5/13 (38.5%), but with 2.4 times lower

Table 4. Association between *IL28B* genotypes and the occurrence of late virological relapse evaluated by logistic regression.

	univariate			
	beta	**P**	**RR***	**95% CI**
$CC_{rs12979860}$	-1.814	*0.013*	0.522	0.278-0.979
$TT_{rs12979860}$	0.821	0.358	2.000	0.462-8.664
$GG_{rs8099917}$	-0.329	0.737	1.333	0.251-7.084
$TT_{rs8099917}$	20.64	0.999	1.167	1.003-1.357
$AA_{rs12980275}$	0.423	0.968	1.000	0.208-4.814

*RR-relative risk.

relative risks (RR = 0.538 CI95% 0.2-1.448 *vs*. RR = 1.289 CI95% 0.807-2.058). However, a time-dependent association between $CC_{rs12979860}$ genotype and the progression of fibrosis was not established (P = 0.519).

Unfavourable clinical outcomes were noted in a total of three cases, during follow up period, including an occurrence of ascites in two and HCC in one patient. The incidence of unfavourable clinical outcomes among patients with $CC_{rs12979860}$ genotype was 2/29 (6.9%) and in non-CC genotype 1/13 (7.7%) (P = 0.926). Relative risks were slightly greater for patients with non-CC genotypes (RR = 1.009 CI 95% 0.089-9.029 *vs*. RR = 0.897 CI 95% 0.089-9.029). There was no significant time-dependent association between $CC_{rs12979860}$ genotype and unfavourable outcome during follow-up (P = 0.764).

Discussion

There is a significant geographical difference in allelic frequencies of the rs12979860 C allele (although it is established as the most common allele worldwide), ranging from the lowest of 38% in African, intermediary (50-85%) in European/Caucasian populations and up to 100% in East Asian population [11]. These host genetic variants offer a plausible explanation for otherwise clinically inexplicable differences in treatment response rates among different ethnicities. Large-scale studies have shown that each population has a relatively unique pattern of gene polymorphism, which may have a significant effect on disease susceptibility, immunogenetics and pharmacogenetics [12]. Reports of the protective $CC_{rs12979860}$ genotype frequencies are also diverse, ranging from 44-49% in a European population, up to 78.58% in East Asian subjects who also have higher reported SVR rates than patients of European ancestry [13,14].

In our study group, the most prevalent genotypes in all three SNP positions were: $CC_{rs12979860}$ genotype in 69% of patients, $GT_{rs8099917}$ in 78.6% of patients and $GG_{rs12980275}$ in 47.6% of patients. These frequencies differ from the only available published data concerning Serbian population by Lazarevic *et al*., who had shown lower frequencies of $CC_{rs12979860}$ genotype-24.5% in the whole study group of 106 patients with genotype 1 HCV infection and in 33.9% of patients who achieved SVR [9]. Among authors from our surroundings, a group of Croatian authors Grgic *et al*. analysed SNP of rs12979860 in 595 patients with CHC genotype 1 and showed predominance of $CT_{rs12979860}$ (56.3 %) genotype, as well as lower frequencies of $CC_{rs12979860}$ (29.1%) and $TT_{rs12979860}$ (14.6%) genotypes, in treatment naïve patients with genotype 1 HCV infection [15]. Our results differ, due to the size and specificity of our patient group and partially selection/inclusion criteria, as we included only patients who had achieved SVR, with HCV genotypes other than genotype 1 (genotypes 2 and 3 were also present), as well as differences in genotyping methods. These advantageous frequencies of favourable genotypes in our study may also explain very high treatment success rates in Serbia approximately 79.7% of end of treatment response (EOT) and 70.5% SVR (60.7% in genotype 1 HCV infection) [16,17].

The association between *IL28B* genotypes and liver injury has been substantially studied but remains inconclusive. In our study group, patients with a favourable $CC_{rs12979860}$ genotype had also a lower degree of necroinflammation and frequency of cirrhosis, in agreement with the data from Falleti *et al*. [18]. Di Marco *et al*. in addition demonstrated that apart from non-$CC_{rs12979860}$ genotypes, patients' age and non-$TT_{rs8099917}$ genotypes were also significantly associated with F3-F4 METAVIR scores [19]. Fabricio-Silva *et al*. observed, in a larger multiethnic study, that $CT_{rs12979860}$ genotype and younger age presented protective factors against inflammatory activity [20]. However, results from other authors have shown contradictory results on associations of $CC_{rs12979860}$ genotype with fibrosis and cirrhosis, as well as the absence of any kind of association [6,18,21]. This is probably due to differences in studies' designs and the fact that liver fibrosis in chronic hepatitis C is multicausal, including host related factors-genetics, age, insulin resistance, body mass index, but also the duration of HCV infection, oxidative stress and environmental factors [22].

We also observed that significantly more patients with a favourable $CC_{rs12979860}$ genotype had lower baseline viral loads (HCV RNA < 800.000 IU/mL), but without a significant genotype-associated difference in average baseline viraemia. A possible effect of *IL28B* genotypes on the baseline viral load is still poorly understood, and only a few authors reported significant associations. Boglione *et al*. reported $TT/CC_{rs12979860}$ genotypes association with a higher baseline viral load (HCV RNA >800.000 IU/mL), and the presence of the G allele at rs8099917 with lower viral loads [23]. In a prospective international cohort study of injectable drug users with acute HCV infection, HCV RNA levels 12 months following infection were independently associated with male gender, *IL28B* $CC_{rs12979860}$ genotype and HCV genotype 1 [24]. The importance of these results remains to be elucidated, as some studies

have refuted the effect of HCV RNA loads on disease progression, but it is probable that high levels of HCV RNA may have an impact on immunological response and hepatic inflammation especially in advanced liver injury [25,26].

The relevance of rs12980275 polymorphism remains to be elucidated, but our results confirmed that patients with AA genotype had more unfavourable baseline characteristics- higher pretreatment levels of ALT and level of necroinflammation. Lazarevic *et al*. had shown that AA genotype in combination with other favourable genotypes has a significant impact on achieving SVR [9]. In patients with this genotype, this may be due to a more vigorous immune response and greater liver injury marked by increased hepatic inflammation and higher serum ALT, but a larger scale study is needed in order to evaluate this hypothesis.

In the DAA era the role of genotyping *IL28B* may be diminished, but in resource-limited countries, particularly in Serbia, where interferon-based treatment is still the only available treatment for CHC, it is useful as a predictor of the natural course of HCV infection and treatment success [26]. In this study, we evaluated its role in patients who had baseline severe liver damage and had undergone successful treatment, but in whom, in our clinical practice, in spite of achieving SVR we anticipate and observe virological relapse and/or progression of liver damage. To our knowledge, these are the first published results concerning this particular group of patients in Serbia and the association of *IL28B* genotypes with any clinical events beyond SVR- long term outcomes and late virological relapse.

SVR is considered a reliable endpoint of CHC treatment, although there are numerous reports of patients with detectable levels of HCV RNA even after achieving SVR [27-29]. However, a minority of authors differentiated a relapse after SVR from reinfection and/or a possible occult HCV infection. A comprehensive systematic review showed that SVR appears durable in the majority of patients at 5 years post-treatment, driven mostly by an increased reinfection risk, with 5-year recurrence rates of 0.95%, 10.67%, and 15.02% in the low-risk, high-risk, and coinfection groups, respectively [30]. Furthermore, published reports of late virological relapses described mostly asymptomatic patients with self-limited viraemias, detected after years of careful monitoring and investigation of de novo elevated liver enzyme levels. Lu *et al.* speculated that this may be due to the fact that in these cases SVR represented HCV suppression, rather than HCV eradication [27]. Unfortunately, in Serbia, there is no involvement in any European monitoring systems of HCV infection and no long-term follow-up studies, except for our own clinical observations and patients' feedback during the past decade. We maintained long-term outpatient monitoring of all patients with severe liver injury, including those who achieved SVR, in spite of guidelines that have changed over the years and mostly advocated cost-effective time-limited follow up and selective screening for HCC. Due to intermittent stock outs, the availability of HCV RNA testing was also often limited.

There is overwhelming evidence that patients with severe fibrosis or cirrhosis are still at high risk of liver-related complications, even beyond SVR and/or regression of fibrosis (or reversal of cirrhosis) [31-33]. Several large scale follow-up studies of treatment-experienced patients, with baseline severe liver injury caused by CHC, have shown that comorbidities, such as diabetes mellitus type 2, high levels GGT, high BMI and non-alcoholic steatohepatitis (NASH), are associated with liver fibrosis progression and development of HCC in cirrhotic patients in spite of antiviral treatment outcome [34,35]. Our clinical policy of long-term follow up of patients with baseline severe liver injury is also supported by results from a Scottish cohort showing that liver-related morbidity in patients who achieved SVR may be caused by recurrent alcoholism and other comorbidities, and suggesting that patients ought to be managed even after completion of successful antiviral treatment [32].

In our study, after a significant follow-up period, reevaluation showed that patients with non-CC genotype had a 6 times greater risk for detectable viraemia and were predominant among late relapsers. We also showed that $CC_{rs12979860}$ genotype was a negative predictor of late relapse. So far there have been no published data concerning this subject, as rs12979860 polymorphism was evaluated only as a predictor of SVR and spontaneous HCV clearance. Reported risk factors for late relapse are mostly due to immunosupression (which was not the case in our patients), but there are no other conclusive predictors. This may be due to the durability of SVR, restrictive guidelines concerning monitoring patients with SVR, loss to follow-up, all resulting in a very limited number of patients for such studies. One of the weaknesses of our study was that we were not able to exclude reinfection by RNA sequencing, as only retesting for virus genotypes was available. Nevertheless, none of the patients had risks for reinfection and none of them were previous or active injectable drug users. The main route of HCV transmission was through a blood

transfusion, a trend in the 1990s, before the routine screening of blood derivatives was introduced.

We did not find a time-dependent association between $CC_{rs12979860}$ genotype and fibrosis progression, in accordance to results by Noureddin *et al*., who had additionally observed an association with hepatic inflammation and clinical outcomes, but not with fibrosis, suggesting that the mechanisms for fibrogenesis are inflammation-independent and multicausal, as previously mentioned [6].

Unfavourable clinical outcomes in our study were very infrequent, occurring in three patients and with no evidence of rs12979860 genotype association, probably due to a small number of patients. Similar to our results, Savino *et al*. in a larger 15-year cohort study of patients with HCV-related cirrhosis consisting of treatment naïve and experienced patients with low SVR, did not confirm any influence of *IL28B* genotypes on the clinical outcome, such as decompensation, hepatocellular carcinoma and death, but without considering virological relapse [36].

As this was a pilot study, major limitations are the sample size and the lack of a healthy control group, for a more reliable population study of *IL28B* genotype frequencies. In the group of patients with late relapse, there was a response-bias, as some of the patients from this group had previously been evaluated and aware of detectable HCV RNA, so they were motivated to respond to our invitation for reevaluation, and may have caused an overestimation of late relapse rates. We used non-invasive methods such as fibroscan and ultrasonography to monitor patients for the progress of fibrosis and liver-related complications, as repeated liver biopsies are not ethically justified.

Conclusion

Careful long-term monitoring of patients with severe baseline liver damage is very important and needs to include both screenings for HCC as well as HCV RNA detection. We demonstrated that, in this group of patients who additionally achieved SVR after interferon-based treatment, *IL28B* $CC_{rs12979860}$ genotype can be useful as a negative predictor of late virological relapse, but not for prediction of fibrosis progression and clinical outcomes. Varying long-term prognosis in patients with HCV-related severe fibrosis and cirrhosis is due to complex multicausal mechanisms and interactions between host genetic factors, virus and environment. A further investigation concerning genetic factors is necessary, so that patients who are under risk for late relapse, complications and unfavourable outcomes, can be identified and reevaluated, especially as they are candidates for new treatment options in the DAA era.

Authors' contributions

Jordovic Jelena, Bojovic Ksenija-study design, writing manuscript, collecting data and patients' clinical follow- up. Simonovic-Babic Jasmina, Delic Dragan -manuscript revision,clinical data collection, patients' clinical follow up. Katanic Natasa, Nikolic Natasa, Urosević Aleksandar, Nestorov Jelena –manuscript drafting. Gasic Vladimir, Kotur Nikola, Zukic Branka, Pavlovic Sonja- revision and manuscript drafting, laboratory analysis. Lazarevic Ivana, Karalic Danijela- manuscript revision and laboratory analysis.

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