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AIMS AND SCOPE

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Retrospective Study

Sustained virologic response to direct-acting antiviral agents predicts better outcomes in hepatitis C virus-infected patients: A retrospective study

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Abstract

BACKGROUND
Direct-acting antiviral agents (DAAs) are extremely effective in eradicating hepatitis C virus (HCV) in chronically infected patients. However, the protective role of the sustained virologic response (SVR) achieved by second- and third-generation DAAs against the onset of hepatocellular carcinoma (HCC) and mortality is less well established.

AIM
To examine the occurrence of HCC or death from any cause in a retrospective-prospective study of patients treated with DAAs.

METHODS
Patients were enrolled from a tertiary academic hospital center for liver disease management that collects subject data mainly from northeastern Italy. The study was conducted in 380 patients (age: 60 ± 13 years, 224 males, 32% with cirrhosis) treated with DAAs with or without SVR (95/5%), with a median follow up of 58 wk (interquartile range: 38-117). The baseline anthropometric features, HCV viral load, severity of liver disease, presence of extra-hepatic complications, coinfection with HIV and/or HBV, alcohol consumption, previous interferon use, alpha-fetoprotein levels, and renal function were considered to be confounders.

RESULTS
The incidence rate of HCC in patients with and without SVR was 1.3 and 59 per 100 person-years, respectively (incidence rate ratio: 44, 95%CI: 15-136, $P < 0.001$). Considering the combined endpoint of HCC or death from any cause, the hazard ratio (HR) for the SVR patients was 0.070 (95%CI: 0.025-0.194, $P < 0.001$). Other

independent predictors of HCC or death were low HCV viremia (HR: 0.808, *P* = 0.030), low platelet count (HR: 0.910, *P* = 0.041), and presence of mixed cryoglobulinemia (HR: 3.460, *P* = 0.044). Considering SVR in a multi-state model, the independent predictors of SVR achievement were absence of cirrhosis (HR: 0.521, *P* < 0.001) and high platelet count (HR: 1.019, *P* = 0.026). Mixed cryoglobulinemia predicted the combined endpoint in patients with and without SVR (HR: 5.982, *P* = 0.028 and HR: 5.633, *P* = 0.047, respectively).

CONCLUSION
DAA treatment is effective in inducing SVR and protecting against HCC or death. A residual risk of HCC persists in patients with advanced liver disease or with complications, such as mixed cryoglobulinemia or renal failure.

Key words: Direct-acting antiviral agents; Hepatitis C virus; Mixed cryoglobulinemia; Sustained virologic response; Cirrhosis; Survival analysis

Core tip: The protective role of a sustained virologic response (SVR) achieved by direct-acting antiviral agents (DAAs) against the chronic consequences of hepatitis C virus (HCV) infection is not well established. We examined the occurrence of hepatocellular carcinoma (HCC) or death from any cause in a retrospective-prospective study at our tertiary academic hospital center for liver disease management. We confirmed that DAA treatment is very effective in inducing SVR in HCV-infected patients and in protecting patients against HCC development or death. However, a residual risk of HCC persists, particularly in patients with advanced liver disease or with extra-hepatic HCV-related complications.

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is highly prevalent in many areas of the world and, because of its serious associated hepatic and extrahepatic complications, it constitutes an important burden for the global health system[1]. Chronic HCV infection can cause both liver disease [cirrhosis and hepatocellular carcinoma (HCC)] and extrahepatic diseases (lymphomas, vasculitis, progressive renal failure) that could lead to patient death[2]. The classic treatment of HCV infection with interferon-based regimens has shown a low efficacy in eradicating HCV infection, particularly in patients with a high grade of liver fibrosis, and cannot be offered to patients with more severe liver disease or several comorbidities due to multiple severe adverse effects[3,4].

New interferon-free therapies based on combinations of direct-acting antiviral agents (DAAs) have progressively improved HCV eradication, granting a sustained virologic response (SVR) in more than 95% of patients with fewer adverse effects than those treated with interferon therapy[5]. However, HCV eradication following treatment with DAAs has not provided clear evidence of a beneficial effect in reducing HCC development in the short term or death in those without advanced liver disease[6]. In fact, some studies have suggested an increased prevalence of early HCC recurrence after treatment with DAAs in patients with a previously cured HCC[7,8].

In Italy, the prevalence of HCV infection is among the highest in Western Europe, reaching 2% of seropositivity and a chronic viremic rate close to 73%[1]. According to the European Medicines Agency guidelines[9], DAAs have been approved by the Italian Medicines Agency (AIFA) since 2014, and they have been made available for HCV-infected patients with advanced liver fibrosis or with significant HCV-related

extrahepatic complications. Since 2017, the treatment has been extended to patients with less advanced liver disease.

In this study, we characterized chronic HCV-infected patients and, after treatment with second- and third-generation interferon-free DAA regimens, we followed the patients for a median of 58 weeks. The aim of this study was to retrospectively analyze the predictors of both SVR and new occurrence of HCC or death after interferon-free DAA treatment. For this purpose, we have used the classic survival analysis and a more sophisticated multi-state survival model where SVR achievement was introduced as a time-dependent variable.

MATERIALS AND METHODS

Patients of any age, sex, and race included in the Registry of Drug Monitoring for DAA use were considered for inclusion in this retrospective study, and the enrollment period lasted from January 2015 to January 2019. The patients were enrolled from a tertiary academic hospital center for liver disease management that collects subject data mainly from northeastern Italy. Patients were included if they had documented HCV chronic viremia and criteria for using DAAs according to the recommendations of the AIFA (Table S1 of the supplemental material) and the European Association for the Study of the Liver (EASL)[10,11]. Patients were excluded from the analysis if, after inclusion, they did not start DAA therapy or did not complete DAA treatment for reasons other than adverse effects. Patients were also excluded if they had incomplete data or were lost to follow up. The names of the DAAs used are specified in Table S2 of the supplemental material.

Some patients in the study had been previously treated with interferon-based regimens without achieving SVR or with adverse effects that precluded the continuation of therapy. These patients were subsequently treated with interferon-free DAAs and were included in the study. The choice of DAAs was made according to the EASL guidelines and under the surveillance of two expert hepatologists. Second- and third-generation DAAs were used with or without ribavirin according to the HCV genotype and inclusion criteria. Treatment was continued for 12 wk, and SVR was defined as undetectable HCV RNA at 12 and 24 wk after the end of therapy. Those patients who did not achieve SVR after 12 wk or did not maintain SVR after 24 wk were considered "nonresponders" or "relapsers", respectively, and were treated again with different genotype-specific DAAs for another 12 wk (rescue treatment). If the first or rescue treatment with DAAs achieved SVR at 12 and 24 wk, the patients were considered "responders"; otherwise, the patients remained "nonresponders".

Liver fibrosis was assessed by either liver biopsy or transient elastography (FibroScan TM®, EchoSens™, Paris, France), and the results were converted to a Metavir score[12]. Cirrhosis was determined by the presence of suggestive clinical signs (altered serum aminotransferases levels, platelet counts, coagulation parameters, gamma-glutamyl-aminotransferase levels, total bilirubin levels, and serum albumin levels) and/or the presence of signs of portal hypertension (ultrasound splenomegaly and endoscopic esophageal or gastric varices), and a Metavir score of F4 (or corresponding Ishak)[13], or a liver stiffness measured by transient elastography higher than 14.5 kPa. Patients with cirrhosis were ranked by the Child-Turcotte-Pugh and MELD scores[14].

All patients were evaluated before starting DAAs and every 3 mo for 1 year by clinical, biochemical, and ultrasound examinations. Thereafter, the follow up was established according to hepatic disease severity. Regular medical controls varied from every 1 to 6 mo per patient. At baseline, we collected general clinical characteristics, anthropometric variables, and hemochromocytometric and coagulation parameters and assessed liver and kidney functions, plasma glucose and alpha-fetoprotein levels and the presence of cryoglobulins. The body mass index was calculated as the body weight divided by the squared height in meters. The glomerular filtration rate was estimated by the Modification of Diet in Renal Disease study equation with 4 variables. The presence of diabetes was documented according to the intake of oral antidiabetic agents and/or the administration of insulin therapy or by fasting blood glucose higher than 125 mg/dL on two separate days. Alcohol consumption was considered when the intake was equal to or greater than 20 g/d of ethanol for both sexes.

The presence of HCC was suspected by ultrasound examination and/or elevated alpha-fetoprotein levels (> 400 ng/mL) and was confirmed by contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI). When doubts persisted, CT-guided liver biopsy was performed. Patients with HCC were staged according to the Barcelona Clinic Liver Cancer system and were treated, whenever

appropriate, by surgical resection according to the Milan criteria[15,16]. Unresectable HCC was treated by liver transplantation, percutaneous lesion radiofrequency or ethanol ablation, or trans-arterial chemoembolization. Patients with HCV viremia and previous HCC that had been treated by surgical resection, liver transplantation, or radical local ablation were considered cured after radiological confirmation (complete radiological response). These patients were treated with DAAs after confirmation of the complete radiological response according to the Italian guidelines and were included in the study. During the follow up, the occurrence of de-novo (new) HCC or death from any cause was registered for all patients until the end of the study. The patient data and informed consent were collected according to Italian laws on observational retrospective studies and the Italian Data Protection Authority. The study was approved by the Institutional Review Board of the University of Udine.

Laboratory and radiological methods

HCV infection was suspected by the detection of serologic anti-HCV antibodies using a standard immunoassay and was confirmed by the measurement of viremia using quantitative RNA analysis (Abbott RealTime HCV, Abbott, Rungis, France). HIV and HBV coinfections were evaluated by standard methods in all HCV-positive patients. The genotype of HCV was established by NS5b gene sequencing[17]. Blood and coagulation variables, plasma aminotransferases, gamma-glutamyl-aminotransferase, alkaline phosphatase, total bilirubin, creatinine, and glucose levels were assessed by automated laboratory methods. The prothrombin time was normalized using standard plasma and was expressed as the international normalized ratio (INR). Plasma albumin was measured using a colorimetric method (ALB2; Roche cobas®) and an automated system (Roche/Hitachi Cobas c 701, Roche Diagnostics GmbH, Mannheim). Cryoglobulins were assessed at baseline according to Vermeersch *et al*[18]. Specifically, the blood was clotted for 1 h, and the serum was separated by centrifugation at 37 °C and refrigerated at 4 °C to allow cryoglobulin precipitation (cryocrit) over 7 d. The cryoglobulin protein concentration was measured by spectrophotometry, and the proteins were characterized after rewarming at 37 °C using an immunologic assay. Only type II or III mixed cryoglobulinemia (MC) was considered potentially linked to HCV infection[2]. We considered a cryocrit greater than 1% to be clinically significant. The plasma alpha-fetoprotein level was measured using an immunoenzymatic chemiluminescence method.

Contrast-enhanced CT and MRI examinations to assess upper abdomen organs were performed by standardized methods using a 64-row multidetector CT scanner (Discovery HD 750, GE Healthcare, Milwaukee, WI, United States) and a 3.0-T magnet using a 32-channel surface coil (Achieva, Philips Medical System, Best, the Netherlands), respectively. Liver biopsies were evaluated by an expert liver histopathologist.

Statistical methods

The data are presented as the means ± standard deviation or medians [interquartile range (IQR)] when appropriate. The normal distribution of variables was assessed by histogram analysis and the Shapiro-Wilks test for normality. Skewed variables were log transformed to achieve a normal distribution. Proportions were presented as counts (%). The mean difference was assessed by student's *t*-test or by the nonparametric Wilcoxon-Mann-Whitney test when appropriate. Proportions were analyzed as contingency tables, and differences were assessed by Fisher's exact test. Correlation analysis was performed by calculating the Pearson's correlation coefficient. The variance in the continuous variables across factor levels was assessed by one-way analysis of variance (ANOVA), and Tukey's method was used to correct for multiple comparisons. The incidence rate and incident rate ratio with 95%CIs were calculated as measures of incidence and for comparative purposes (analyzed by a chi-Squared test). Kaplan-Meier curves with a 95%CI and frequency tables were used to represent the event rates over time, and the nonparametric log-rank test was used to assess significant differences. Factors associated with the event rate were analyzed by Cox's proportional hazards analysis in univariate and multivariate models, and the results were expressed as hazard ratios (HRs) at 95%CIs. To analyze the predictors of SVR achievement and the predictors of HCC or death occurrence with or without SVR achievement, multi-state analysis with 3 states (Figure 1) was conducted. In this model, SVR was considered as a time-dependent variable, and Cox's proportional hazards were measured for each of the 3 state transitions using a clock-forward approach in a Markov chain model (Cox-Markov model)[19]. The multi-state Cox-Markov model is an extension of the classical survival analysis; with this model, we investigated the effect of different baseline cofactors for each disease state[20].

Multivariate model selection was assessed by the evaluation of the best model starting from a model that included age and sex and considered all other variables
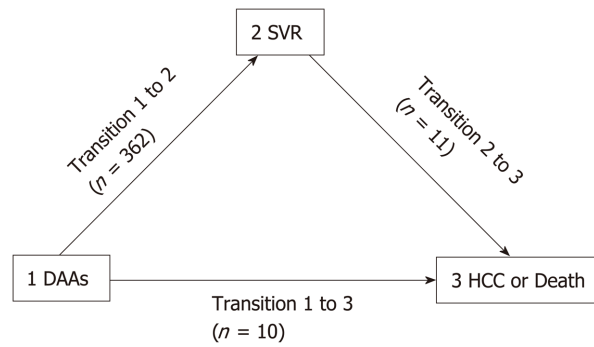
Figure 1 Multi-state transition model. DAAs: Direct-acting antiviral agents; SVR: Sustained virologic response; HCC: Hepatocellular carcinoma.

with univariate statistical or biological relevance by a stepwise forward approach based on the Akaike information criterion[21]. For statistical analysis, we used software R (The R Foundation for Statistical Computing, Vienna, Austria, version 3.5.2) using the "epiR" (version 0.9-99), "survival" (version 2.43-3), "mstate" (version 0.2.11), and "MASS" (version 7.3-51.1) packages[22].

RESULTS

The study cohort comprised 399 patients who were prescribed interferon-free DAAs. Two of the patients did not initiate DAAs because of alcohol abuse or decompensated cirrhosis, two patients did not complete DAA treatment because they did not comply with the therapy, and 15 patients were lost to follow up or had incomplete data. The remaining 380 patients were included in the analysis. Cirrhosis was diagnosed in 122 patients before starting DAA treatment. Eight patients had a previous diagnosis of HCC that was cured by orthotopic liver transplantation in six patients, and by surgical resection or percutaneous radiofrequency in the remaining two patients. We observed no significant interactions between DAAs and immunosuppressive agents that led us to modify the dose or drug type. The frequencies of the different interferon-free DAAs used and the genotypes of HCV are reported in Supplemental Tables S2 and S3, respectively. DAA treatment was effective in 362 patients (95%), and the overall incidence rate of HCC was 3.2 per 100 person-years. The incidence rate of HCC in patients who did not achieve SVR was 59 per 100 person-years, whereas it was 1.3 per 100 person-years in those with SVR (incidence rate ratio: 44, 95%CI: 15-136, $P < 0.001$). Five patients interrupted DAA treatment for adverse events, 1 in the group with SVR and 4 in the group without SVR ($P < 0.001$). The frequency of different adverse events reported during treatment is presented in Supplemental Table S4.

At baseline, the patients with SVR had a higher HCV viral load, platelet count, and plasma albumin levels than the patients without SVR, and the patients with SVR a lower occurrence of previous HCC, prevalence of cirrhosis, relapse of HCV infection after initial DAA treatment, and lower Metavir scores, INR, and plasma alpha-fetoprotein levels (Table 1) than the patients without SVR. Patients who developed HCC during the follow up after DAA therapy had, at baseline, a higher occurrence of previous HCC, relapse of HCV infection after initial DAA treatment, presence of plasma cryoglobulins, higher Metavir score, and prevalence of cirrhosis, INR, and plasma alpha-fetoprotein levels; these patients also had a lower HCV viral load, plasma albumin levels, and platelet count than patients without HCC (Table 1).

The baseline age, sex, BMI, alcohol intake, presence of diabetes, renal function, use of ribavirin in association with DAAs, previous use of interferon and plasma total bilirubin levels were not associated with the achievement of SVR or the development of HCC (Table 1). Although a different proportion between the type of inclusion criteria and presence of HCC was observed, no differences were seen between the inclusion criteria and SVR and between the HCV genotype and presence of SVR or HCC (Supplemental Table S3), whereas a difference in the proportion between the DAAs used and the presence of SVR or HCC was observed ($P = 0.010$ and $P < 0.001$, respectively; Supplemental Table S2). DAA treatment induced SVR similarly in patients who had previously used interferon and in those who had not, at rates of 94.8% and 95.5%, respectively. HCC developed in 5.8% or 3.7% of patients who had used interferon or not previously, respectively. The differences between these groups

Table 1 Characteristics of the patients treated for hepatitis C virus infection with direct-acting antiviral agents according to the sustained virologic response and hepatocellular carcinoma development during follow up

Variable	All patients (*n* = 380)	No SVR (*n* = 18)	SVR (*n* = 362)	*P* value	No HCC (*n* = 363)	HCC (*n* = 17)	*P* value
General clinical characteristics							
Age (yr)	60 ± 13	62 ± 10	59 ± 13	NS	59 ± 13	61 ± 9	NS
Male, *n* (%)	224 (58.9)	14 (77.8)	210 (58.0)	NS	213 (58.7)	11 (64.7)	NS
BMI (kg/m^2)	24.9 ± 3.7	24.6 ± 2.7	24.9 ± 3.7	NS	24.9 ± 3.7	24.2 ± 3.2	NS
Alcohol use, *n* (%)	17 (4.5)	2 (11.1)	15 (4.1)	NS	16 (4.4)	1 (5.9)	NS
Diabetes, *n* (%)	48 (12.6)	4 (22.2)	44 (12.1)	NS	44 (12.1)	4 (23.5)	NS
Plasma creatinine (mg/dL)	0.86 ± 0.24	1.03 ± 0.42	0.86 ± 0.23	NS	0.86 ± 0.24	0.97 ± 0.30	NS
eGFR (mL/min/1.73 m^2)	87 ± 25	91 ± 18	87 ± 25	NS	87 ± 25	86 ± 16	NS
HCV infection-related variables							
HCV viremia (*n* × 10^3)	956 (203-2944)	297 (47-1442)	977 (219-3059)	0.033	967 (219-3049)	244 (36-1864)	NS
HIV coinfection, *n* (%)	8 (2.1)	0	8 (100)	-	8 (100)	0	-
Previous use of interferon, *n* (%)	137 (36.0)	7 (38.9)	130 (35.9)	NS	129 (35.5)	8 (47.1)	NS
Use of ribavirin association, *n* (%)	89 (23.4)	5 (27.8)	84 (23.2)	NS	84 (23.1)	5 (29.4)	NS
SVR, *n* (%)	362 (95.3)	-	362 (100)	-	355 (97.8)	10 (58.8)	< 0.001
Time to SVR (wk)	12 (12-13)	-	12 (12-13)	-	12 (12-12)	15 (13-23)	< 0.001
HCV relapse, *n* (%)	20 (5.3)	12 (66.7)	8 (2.2)	< 0.001	13 (3.6)	7 (41.2)	< 0.001
Mixed cryoglobulinemia, *n* (%)	35 (9.2)	4 (22.2)	31 (8.6)	NS	30 (8.3)	5 (29.4)	0.014
Liver structure and function variables							
Metavir score	2.6 ± 1.2	3.7 ± 0.6	2.6 ± 1.2	< 0.001	2.6 ± 1.2	3.9 ± 0.3	< 0.001
Cirrhosis, *n* (%)	122 (32.1)	14 (77.8)	108 (29.8)	< 0.001	107 (29.5)	15 (88.2)	< 0.001
Plasma albumin (g/L)	41.0 ± 5.4	37.6 ± 6.3	41.2 ± 5.3	0.027	41.4 ± 5.4	38.7 ± 5.0	0.047
Plasma total bilirubin (g/dL)	0.79 ± 0.41	0.92 ± 0.50	0.78 ± 0.41	NS	0.78 ± 0.41	0.94 ± 0.47	NS
Plasma alpha-fetoprotein (ng/mL)	4.10 (2.87-7.10)	6.30 (5.20-8.70)	4.10 (2.75-7.05)	0.012	4.10 (2.70-6.80)	8.00 (4.95-10.80)	0.001
Platelet count (*n* × 10^3/mL)	167 ± 67	114 ± 67	170 ± 66	0.003	170 ± 66	98 ± 62	< 0.001
INR	1.05 ± 0.12	1.14 ± 0.19	1.04 ± 0.11	0.038	1.05 ± 0.11	1.17 ± 0.17	0.013
HCC-related variables							
Previous HCC, *n* (%)	8 (2.1)	3 (16.7)	5 (1.4)	0.004	5 (1.4)	3 (17.6)	0.004
HCC, *n* (%)	17 (4.5)	10 (55.5)	7 (1.9)	< 0.001	-	17 (100)	-
Time to HCC (wk)	26 (17-65)	25 (12-57)	40 (25-63)	NS	-	26 (17-65)	-
Death, *n* (%)	8 (2.1)	3 (16.7)	5 (1.4)	0.004	4 (1.1)	4 (23.5)	< 0.001
Time to death (wk)	65.5 (50.5-126.8)	40 (28-79)	76 (55-150)	NS	65 (55-95)	79 (34-127)	NS

HCV: Hepatitis C virus; SVR: Sustained virologic response; HCC: Hepatocellular carcinoma; BMI: Body mass index; eGFR: Estimated glomerular filtration rate; NS: Not significant; INR: International standardized ratio; HIV: Human immunodeficiency virus.

were not significant. Three of eight patients with a previously cured HCC showed recurrence of HCC, and all 3 of these patients did not achieve SVR after DAA treatment. The remaining 5 patients achieved SVR and did not develop HCC. The log of the HCV viral load was directly associated with the log of the platelet count (*P* = 0.014), and a significant difference was found between the log HCV viral load across Metavir scores (*P* = 0.014); specifically, the viral load in the Metavir F4 score was significantly lower than that in the F3 score (*P* = 0.032 after Tukey's correction). An inverse relationship was found between the platelet count across Metavir scores (*P* < 0.001).

Patients with MC were more frequently of the female sex (95% *vs* 83%, *P* < 0.001), older (64 ± 10 *vs* 59 ± 13 years, *P* = 0.005), and with a worse renal function (eGFR 79 ± 24 *vs* 88 ± 25 mL/min/1.73 m², *P* = 0.039) than patients without MC at baseline. No association was found between MC and the Metavir score, presence of cirrhosis, platelet count, albumin levels or INR. HIV coinfection was not associated with SVR or HCC. No HBV coinfection was detected in all included patients.

The effect of SVR on the combined endpoint of a new HCC occurrence or death from any cause during the follow up is represented by Kaplan-Meier curves in Figure 2. In a median follow up of 58 wk (IQR 38-117), the patients with SVR had a higher

survival rate free from HCC than patients who did not achieve SVR (HR: 0.035, 95%CI: 0.015-0.084, *P* < 0.0001, Figure 2). In classical Cox's multivariate proportional hazards analysis, the factors that best and independently predicted the combined endpoint were the log of HCV viremia (HR: 0.808, 95%CI: 0.666-0.980, *P* = 0.030), platelet count (HR: 0.910, 95%CI: 0.831-0.996, *P* = 0.041), presence of MC (HR: 3.460, 95%CI: 1.035-11.56, *P* = 0.044), and presence of SVR (HR: 0.070, 95%CI: 0.025-0.194, *P* < 0.001). The predictors were independent of age, sex, renal function, and presence of cirrhosis (Table 2). In Figure 3, we reported the independent effect of both the SVR and MC status on the combined endpoint of HCC or death from any cause. The effect of the presence of cirrhosis on the combined end-point by SVR achievement is represented in Figure S1 of the supplemental material.

Since the baseline hazards did not satisfy the proportional hazards assumption in the multi-state Cox-Markov model, we used stratified hazards instead of proportional hazards to assess baseline hazards for each separate state transition, as shown in Figure 1 (Table 3). The analysis showed that the best and independent predictors of SVR achievement (transition 1→2) were the absence of cirrhosis (HR: 0.521, 95%CI: 0.404-0.672, *P* < 0.001) and higher platelet count (HR: 1.019, 95%CI: 1.002-1.036, *P* = 0.026); the predictor of HCC or death in patients who did not achieve SVR (transition 1→3) was the presence of MC (HR: 5.633, 95%CI: 1.022-31.05, *P* = 0.047); and the predictors of HCC or death of patients who achieved SVR (transition 2→3) were the presence of MC (HR: 5.982, 95%CI: 1.217-29.41, *P* = 0.028), lower platelet count (HR: 0.958, 95%CI: 0.939-0.979, *P* < 0.001), and lower eGFR (HR: 0.948, 95%CI: 0.915-0.985, *P* = 0.005).

DISCUSSION

In this study, HCV-infected patients who achieved SVR after DAA treatment had a significantly higher survival rate free from new HCC occurrence and a lower rate of death from any cause than patients who did not achieve SVR. This association was strongly independent of confounders in a classical survival model where the presence of SVR was considered a baseline variable together with age, sex, HCV viremia, presence of cirrhosis, MC, platelet count, and renal function.

Although SVR achievement using interferon-based regimens is a predictor of HCC-free survival[23], similar evidence is less clear for DAAs. Different retrospective studies have reported that treatment with these agents did not reduce the incidence of HCC[7] but can even increase the risk of early HCC recurrence after treatment[7,8]. Conversely, other studies with larger samples have suggested a strong beneficial effect of SVR on both the occurrence and recurrence of HCC[24-27]. Our study demonstrated that the achievement of SVR is robustly associated with a better HCC-free survival than a lack of SVR and is independent of previous interferon-based regimen use. However, the lack of a control group precluded the possibility of testing whether DAA treatment per se can be a risk factor for HCC development, although the overall observed HCC incidence rate was similar to that in previous studies with different HCV treatments[6]. Notably, it should be considered that patients with fibrosis > F2 (Metavir) or cirrhosis that achieved SVR after DAA treatment should still need surveillance for their residual risk to develop HCC[28].

SVR is a condition more frequently achieved in HCV-infected patients with a lower grade of liver fibrosis and less advanced hepatic disease. Patients with cirrhosis and/or a decompensated liver disease are less prone to achieve SVR, and these patients are also those with the highest probability of developing HCC and dying due to several causes[29]. Additionally, in our study, the predictors of SVR achievement were a low Metavir score or the absence of cirrhosis and a high platelet count, indicating the expression of less advanced liver disease. In addition, in patients who achieved SVR, the signs of portal hypertension and impaired renal function, such as the markers of advanced liver disease, are independent predictors of HCC development and death from any cause. This is an important point, since the use of DAAs was only recently approved in Italy for HCV patients with less advanced hepatic disease. In those low-risk patients, the efficacy of HCV eradication on the prevention of HCC was indeed more pronounced. This extension of DAAs use is expected to significantly reduce the HCV epidemic and the global burden of HCV-related complications.

In our study, a low baseline viral load was a predictor of HCC development or death from any cause. This point has been explored by Duvoux *et al*[30] who evaluated the HCV viral load in 3 groups of patients with chronic HCV infection: (1) Without cirrhosis; (2) With compensated cirrhosis; and (3) With end-stage cirrhotic disease waiting for liver transplantation[30]. The HCV viral load was significantly lower in

Table 2 Cox's proportional hazards analysis for the combined endpoint of hepatocellular carcinoma occurrence or death from any cause

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (yr)	1.003 (0.968-1.040)	0.862	1.008 (0.958-1.060)	0.760
Male (no/yes)	1.334 (0.538-3.308)	0.534	2.492 (0.683-9.089)	0.167
HCV viremia (log *n*)	0.811 (0.698-0.944)	0.007	0.808 (0.666-0.980)	0.030
Cirrhosis (no/yes)	9.259 (2.705-31.70)	< 0.001	3.392 (0.861-13.37)	0.081
Platelet count ($n \times 10^4$/mL)	0.806 (0.735-0.885)	< 0.001	0.910 (0.831-0.996)	0.041
eGFR (10 mL/min/1.73 m^2)	0.935 (0.780-1.122)	0.473	0.774 (0.596-1.005)	0.055
Mixed cryoglobulinemia (no/yes)	4.522 (1.822-11.23)	0.001	3.460 (1.035-11.56)	0.044
SVR (no/yes)	0.035 (0.015-0.084)	< 0.001	0.070 (0.025-0.194)	< 0.001

HCV: Hepatocellular C virus; SVR: Sustained virologic response; HR: Hazard ratio; CI: Confidence interval; eGFR: Estimated glomerular filtration rate.

end-stage cirrhosis disease than in the other groups and, more interestingly, the viral load increased after liver transplantation. This inverse relationship occurred despite the HCV genotype. The authors concluded that HCV replication is lower in end-stage cirrhotic disease because of a reduced hepatocyte mass and/or an impaired cell-to-cell virus transmission probably due to extensive liver fibrosis[30]. These HCV chronic patients were also those with the lowest probability of achieving SVR and highest risk of developing HCC or dying from any cause. In our study, the inverse association between the HCV viral load and severity of liver disease has been suggested by the indirect relationship between the viral load and Metavir score, and the direct relationship between the viral load and platelet count. However, the role of HCV viremia as a predictive factor of HCC development or mortality in cirrhotic patients remains controversial[31].

Interestingly, we also observed that the baseline presence of an MC predicted the probability of developing HCC or dying from any cause independently of SVR achievement. Asymptomatic or symptomatic MC is associated with HCV viral infection by mechanisms that are not well understood. Generally, stimulation of the B-lymphocyte system by HCV facilitates the development of immortalized B-cell clones releasing IgM with rheumatoid factor activity and the formation of immunocomplexes between immunoglobulins and HCV molecular components that precipitate in plasma below 37 °C. These immunocomplexes activate complement, inducing vascular inflammation that leads to vascular inflammation and cryoglobulinemic syndrome[32]. It is not clear how the presence of cryoglobulinemia could influence SVR achievement and/or HCC development. The study of Passerini *et al*[33] showed no difference in SVR achievement at 12 wk after DAA treatment between HCV patients with and without serum cryoglobulins; however, a previous meta-analysis of 19 observational studies, with more than 2000 patients enrolled, demonstrated that the development of cirrhosis was 5 times as frequent in patients with MC as in those without (OR: 4.90, 95%CI: 3.32-7.15)[34]. Paradoxically, the long-term prospective study of Lauletta *et al*[35] showed that the presence of a cryoglobulinemic syndrome is an independent protective factor against the development of both cirrhosis and HCC, although no difference was found in the overall survival between patients with or without cryoglobulins in 15 years of observation[35]. Nevertheless, the prevalence of MC in our study was lower than that expected from previous studies (40%-60%)[33], likely because of our elevated cut-off point for MC diagnosis that could have led us to underestimate low-grade MC[18].

This study has several limitations that need to be highlighted. First, we included all patients who had been treated with DAAs at a single center, without selection criteria to increase the sample size and improve the statistical power of the study. However, the study population was heterogeneous, and several unknown confounders could have influenced the results. Thus, we have used statistical adjustments in inferential methods and performed multivariate analyses with significant and clinically important confounders, factoring in such heterogeneity as much as possible. Second, we had a low incidence of events and a low median observational time. This low incidence is because we included only HCV patients treated with second- and third-generation DAAs that were introduced in Italy only recently. This point is important because the low outcome rate together with the elevated SVR achievement rate could
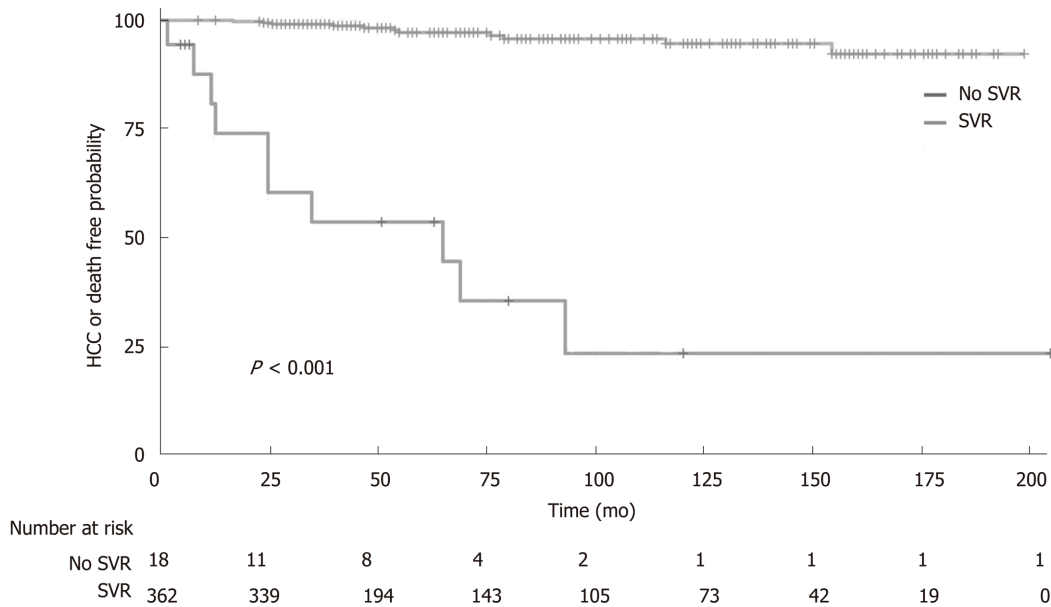
Figure 2 Kaplan-Meier curves and risk table representing survival free of hepatocellular carcinoma or death from any cause according to sustained virologic response to hepatitis C virus direct-acting antiviral agents. Plain areas represent the 95% confidence interval. *P* value by the nonparametric log-rank test. SVR: Sustained virologic response; HCC: Hepatocellular carcinoma.

have limited the power of our multi-state Cox-Markov analysis to confirm a protective effect of SVR on HCC development and mortality. On the other hand, the strength of our study is that the use of a multi-state Cox-Markov model has permitted us to explore more deeply the relationships of the baseline variables with events in each different disease state, which is not possible with a classical survival Cox model. Third, the lack of a control group did not permit exploration of the hypothesis of DAAs as a potential risk factor for HCC development; however, this investigation was not among the aims of the study. Fourth, the follow up interval could be relatively short for HCC development, at least in a general population of HCV-infected patients. However, because the national guidelines limits DAA prescription to patients with advanced or complicated liver disease (Table S1 of supplemental material), we necessarily treated patients with a high risk of developing HCC in a shorter period.

In conclusion, we confirmed that DAA treatment is highly effective in eradicating HCV infection, and this eradication occurs more frequently in patients with less advanced liver disease. In addition, HCV-infected patients who achieved SVR after DAA treatment have a higher survival free from new HCC occurrence or death from any cause than patients who did not achieve SVR. In a classical survival model, this association was strongly independent of confounders.

Table 3 Multi-state Cox-Markov stratified hazards model for transitions in Figure 1

Variable	HR	95%CI	*P* value
Transition 1→2			
Age (yr)	0.997	0.989-1.005	0.493
Male (no/yes)	0.789	0.615-1.013	0.062
HCV viremia (log *n*)	0.983	0.940-1.029	0.470
Cirrhosis (no/yes)	0.521	0.404-0.672	< 0.001
Mixed cryoglobulinemia (no/yes)	0.755	0.520-1.098	0.141
Platelet count ($n \times 10^4$/mL)	1.019	1.002-1.036	0.026
eGFR (10 mL/min/1.73 m^2)	1.003	0.998-1.008	0.235
Transition 1→3			
Age (yr)	0.975	0.912-1.043	0.466
Male (no/yes)	2.040	0.249-16.74	0.507
HCV viremia (log *n*)	0.771	0.589-1.009	0.058
Cirrhosis (no/yes)	5.704	0.620-52.51	0.124
Mixed cryoglobulinemia (no/yes)	5.633	1.022-31.05	0.047
Platelet count ($n \times 10^4$/mL)	0.928	0.981-1.005	0.229
eGFR (10 mL/min/1.73 m^2)	1.007	0.970-1.045	0.726
Transition 2→3			
Age (yr)	1.019	0.945-1.098	0.625
Male (no/yes)	3.613	0.692-18.88	0.128
HCV viremia (log *n*)	0.972	0.715-1.321	0.857
Cirrhosis (no/yes)	2.720	0.479-15.44	0.259
Mixed cryoglobulinemia (no/yes)	5.982	1.217-29.41	0.028
Platelet count ($n \times 10^4$/mL)	0.958	0.935-0.982	< 0.001
eGFR (10 mL/min/1.73 m^2)	0.948	0.915-0.985	0.005

HCV: Hepatitis C virus; HR: Hazard ratio; CI: Confidence interval; eGFR: Estimated glomerular filtration rate.

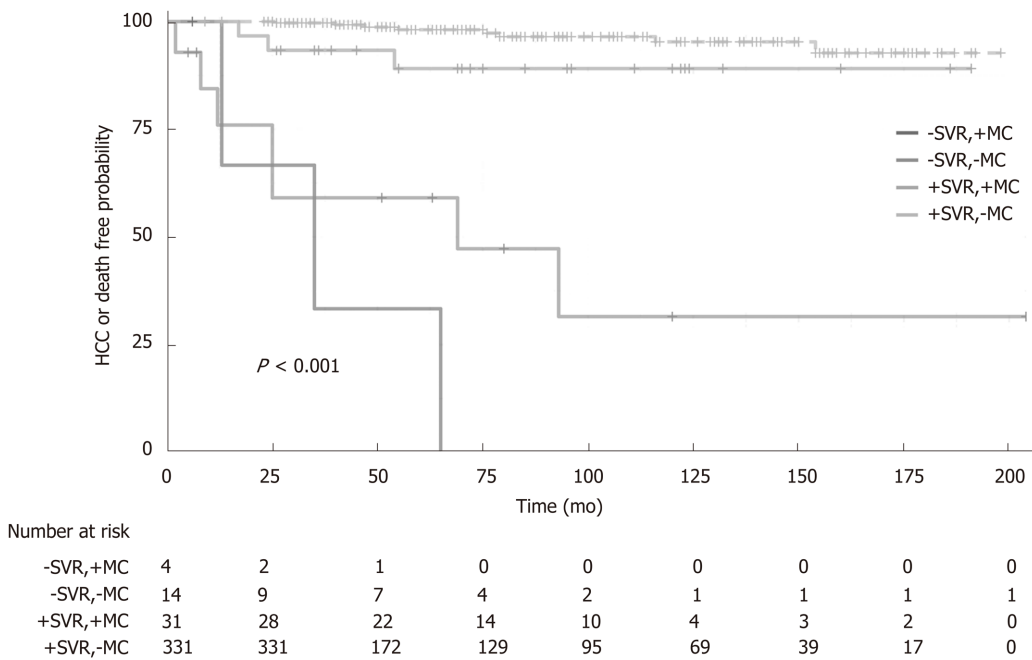**Figure 3 Kaplan-Meier curves and risk table representing survival free of hepatocellular carcinoma or death from any cause according to the presence of mixed cryoglobulinemia and sustained virologic response to hepatitis C virus direct-acting antiviral agents.** *P* value by nonparametric log-rank test. +: with; -: without; MC: Mixed cryoglobulinemia; SVR: Sustained virologic response; HCC: Hepatocellular carcinoma.

ARTICLE HIGHLIGHTS

Research background

Direct-acting antiviral agents (DAAs) are extremely effective in eradicating hepatitis C virus (HCV) in chronically infected patients. However, the protective role of a sustained virologic response (SVR) achieved by second- and third-generation DAAs against the onset of hepatocellular carcinoma (HCC) and mortality is less well established.

Research motivation

The main topic of this study was to establish the effectiveness of new generations of DAAs to induce SVR and to prevent HCC development or mortality in HCV-infected patients. These patients were characterized by an elevated risk for HCC because of their advanced or complicated liver disease. The strength of this study was the use of a multi-state Cox-Markov model. This model permitted us to explore more deeply the relationships of baseline variables with events in each different disease state. This last approach is not possible with a classical survival Cox model.

Research objectives

The aim of this study was to examine HCC occurrence or death from any cause in a retrospective-prospective study of patients treated with DAAs and to identify potential predictors of SVR achievement and HCC development.

Research methods

The patients were enrolled from a tertiary academic hospital center for liver disease management that collects subject data mainly from northeastern Italy. The study was conducted using 380 patients (age: 60 ± 13 years, 224 males, 32% with cirrhosis) treated with DAAs with or without SVR (95/5%), with a median follow up of 58 wk (interquartile range: 38-117). The baseline anthropometric features, HCV viral load, severity of liver disease, presence of extra-hepatic complications, coinfection with HIV and/or HBV, alcohol consumption, previous interferon use, alpha-fetoprotein levels, and renal function were considered to be confounders. Survival analysis was conducted using a classical Cox model and a more advanced Cox-Markov multi-state model that considered SVR as a time-dependent variable that can influence the probability of the outcome.

Research results

The incidence rates of HCC in patients with and without SVR were 1.3 and 59 per 100 person-years, respectively (incidence rate ratio: 44, 95%CI: 15-136, $P < 0.001$). Considering the combined endpoint of HCC or death from any cause, the hazard ratio (HR) for SVR patients was 0.070 (95%CI: 0.025-0.194, $P < 0.001$). Other independent predictors of HCC or death were low HCV viremia (HR: 0.808, $P = 0.030$), low platelet count (HR: 0.910, $P = 0.041$), and presence of mixed cryoglobulinemia (HR: 3.460, $P = 0.044$). Considering SVR in a multi-state model, independent predictors of SVR achievement were the absence of cirrhosis (HR: 0.521, $P < 0.001$) and high platelet count (HR: 1.019, $P = 0.026$). Mixed cryoglobulinemia predicted the combined endpoint in patients with and without SVR (HR: 5.982, $P = 0.028$ and HR: 5.633, $P = 0.047$, respectively).

Research conclusions

Treatment with DAAs is very effective in inducing SVR and protecting against HCC or death. A residual risk for HCC persists in patients with advanced liver disease or disease complicated by extra-hepatic manifestations, such as mixed cryoglobulinemia and renal failure.

Research perspectives

New generations of DAAs should be recommended to treat all patients with HCV infection independently of the status of their liver disease or the presence of extra-hepatic manifestations. Further studies are needed to define a better strategy to treat HCV-infected patients with more advanced or complicated liver disease.

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