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ORIGINAL ARTICLE

Vitamin D supplementation improves response to antiviral treatment for recurrent hepatitis C

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Summary

In immune-competent patients, higher vitamin D levels predicted sustained viral response (SVR) following interferon (INF) and ribavirin therapy for chronic hepatitis C. This study aimed to verify the influence of vitamin D serum levels and/or vitamin D supplementation in predicting SVR rates for recurrent hepatitis C (RHC). Forty-two consecutive patients were treated for RHC with combination therapy with INF-α and ribavirin for 48 weeks. Vitamin D serum levels were measured in all patients before antiviral therapy. In 15 patients oral vitamin D3 supplementation was administered to avoid further bone loss. SVR was observed in 13 patients; it was achieved in 1/10 severely vitamin D deficient (≤10 ng/ml) patients, in 6/20 deficient (>10 and ≤20 ng/ml) and in 6/12 with near normal (>20 ng/ml) 25-OH vitamin D serum levels ($P < 0.05$). Cholecalciferol supplementation, in the presence of a normal or near normal baseline vitamin D concentration, (improvement of chi-square $P < 0.05$, odds ratio 2.22) and possessing a genotype other than 1 (improvement of chi-square $P < 0.05$, odds ratio 3.383) were the only variables independently associated to SVR. In conclusion, vitamin D deficiency predicts an unfavourable response to antiviral treatment of RHC. Vitamin D supplementation improves the probability of achieving a SVR following antiviral treatment.

Introduction

Hepatitis C virus (HCV) related liver cirrhosis is the most common indication for liver transplantation (LT) worldwide [1,2]. Recurrence of hepatitis C in the graft is almost universal [3] and it is well documented that approximately one third of patients develops graft cirrhosis 5 years after LT. In this setting, graft cirrhosis is associated with a high rate of clinical decompensation, which represents the most common cause of death in this category of recipients [4,5]. The overall impact of HCV recurrence is mirrored in the worse outcome of LT in HCV positive patients when compared with other aetiologies [6]. Therefore, the management of recurrent hepatitis C (RHC) represents one of the most challenging topics in the field of LT [7].

The cornerstone of RHC treatment is combination therapy with standard or pegylated interferon (INF) plus ribavirin for 48 weeks. This treatment is aimed to obtain a sustained viral response (SVR), which is defined as undetectable serum HCV RNA 24 weeks after the stop of antiviral therapy. Achievement of SVR is clearly associated with a better graft and recipient survival. Three different strategies of antiviral treatment can be chosen: prophylactic treatment [8], early pre-emptive treatment [9] and treatment of the established HCV recurrent hepatitis [10],

the latter associated with the highest rates of SVR. Unfortunately, SVR rates in RHC are worse compared to what occurs in immune-competent patients [11–14]. In fact, patients with RHC present more frequently the well known worse predictors of SVR such as older age, use of immune-suppressive drugs, less common adherence to the antiviral therapy schedule, higher viral load and higher frequency of HCV genotype 1. Furthermore, antiviral treatment may favour the development of acute allograft rejection, which occurs, in some studies, in up to 30% of patients, particularly in those treated with INF mono-therapy [15]. As in the nontransplant setting, SVR rates are higher in HCV genotypes 2/3 infections compared to genotypes 1/4 [16,17]. Some, but not all studies, suggested that SVR is more likely achieved when the immune-suppressive regimen is cyclosporine A-based rather than tacrolimus-based [12,13,16–18].

Vitamin D, beyond its known role in calcium and bone metabolism, possesses important immune functions, favouring innate immunity response and cell differentiation [19]. Recent studies have highlighted the role of vitamin D in two different categories of patients with liver disease. Considering liver transplanted patients, low serum levels of 25-OH vitamin D at time of LT were found to predict acute cellular rejection, whilst daily supplementation with cholecaciferol could prevent it [20]. In immune-competent patients, higher vitamin D levels at baseline were able to predict SVR achievement following INF and ribavirin combination therapy for chronic hepatitis C [21].

No data are present in the literature dealing with the influence of serum vitamin D levels and/or vitamin D supplementation on SVR rates for RHC. Thus, the aim of this retrospective study was to investigate the relationship between serum levels of vitamin D and vitamin D supplementation and the achievement of SVR amongst recipients who underwent combination antiviral therapy for RHC.

Materials and methods

Patients

Data from 89 consecutive patients who underwent LT for HCV-related end stage liver disease at our Institution from 1996 to 2006 and who survived at least 1 month after transplant were retrospectively analyzed. The flow chart of the study is presented in Fig. 1. The study was conducted in accordance to the declaration of Helsinki and approved by our ethical review committee. Between June 1999 and June 2008, 42 patients were treated for RHC. None of these patients had HIV or HBV co-infection. Eligibility criteria for treatment were: detectable serum HCV RNA, persistent ALT elevation, histological evidence of lobular hepatitis consistent with recurrent HCV infection with the presence, in the vast majority of the cases, of liver fibrosis, no signs of acute or chronic rejection, biliary obstruction or ischaemic damage. In three recipients antiviral therapy was started in the absence of liver fibrosis since two of them presented a severe cryoglobulinemic HCV related syndrome and one had a severe HCV related liver steatosis. Cytomegalovirus (CMV) infection was ruled out by absence of CMV antigenemia and CMV inclusion bodies on liver biopsies. All patients had a minimum follow-up of at least 18 months after the start of antiviral therapy. The main characteristics of the studied population are outlined in Table 1.

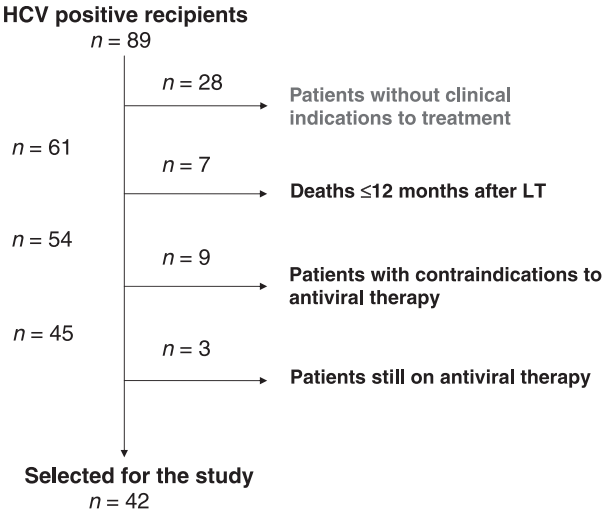

Figure 1 Flow chart summarizing the selection process of the studied population. HCV: hepatitis C virus, LT: liver transplantation.

Immune-suppressive therapy

Immune-suppressive regimen included either cyclosporine or tacrolimus, associated, in the first few months after LT, to corticosteroids. Cyclosporine dosage was calculated to obtain serum levels (measured 2 h after the drug administration) ranging from 800 to 1200 μg/l in the first six weeks after transplant and from 600 to 800 μg/l thereafter. Tacrolimus dosage was calculated to obtain predose serum levels ranging from 10 to 15 μg/l in the first six weeks after transplant and from 5 to 10 μg/l thereafter. Corticosteroid therapy was adopted with a schedule aiming to complete steroid withdrawal by the end of the fourth postoperative month.

Antiviral therapy

All patients were treated with a combination therapy of INF-α and ribavirin. Three different types of INF-α were

Table 1. Baseline clinical and demographic characteristics of the studied population (n = 42). Patients are divided in two groups: those supplemented and those not supplemented with cholecalciferol. Categorical variables are presented as frequencies (%) and continuous variables are presented as medians (range). The statistical analysis was performed using the Pearson chi-square test for categorical variables and Mann–Whitney test for continuous variables.

	Vitamin D		
	Supplemented (n = 15)	Not supplemented (n = 27)	P
Recipient male gender	9 (60%)	21 (77%)	NS
Recipient age (years)	56 (42–61)	52 (23–67)	NS
Donor male gender	11 (73%)	20 (74%)	NS
Donor age (years)	50 (14–68)	39 (18–69)	NS
Body mass index (kg/m^2)	25 (21–29)	24 (20–31)	NS
Days between LT and antiviral therapy	455 (169–3830)	455 (16–2250)	NS
Baseline HCV RNA (IU/ml), $\times 10^3$	759 (0.65–2.730)	522 (0.67–2.768)	NS
HCV genotype 1	12 (80%)	20 (74%)	NS
Tacrolimus-based regimen	10 (66%)	20 (74%)	NS
Length of steroid therapy (days)	152 (30–365)	120 (0–392)	NS
Ishak grading score	4 (1–8)	4 (1–6)	NS
Ishak staging score	1 (0–5)	1 (0–5)	NS
Baseline 25-OH vitamin D > 20 ng/ml	7 (47%)	5 (19%)	P = 0.05
Use of PEG-interferon α-2b	9 (60%)	15 (55%)	NS

LT, liver transplantation.

adopted: (i) standard INF-α2b (Intron-A; Schering Plough, Corp, Kenilworth, NJ, USA) administered subcutaneously at a dose of 3 MU thrice a week in nine patients (21.4%); (ii) leucocyte INF-α (Alfa Wassermann S.p.a., Bologna, Italy) administered subcutaneously at a dose of 6 MU thrice a week in six patients (14.3%); PEG-INF-α2b (PEG-Intron; Schering-Plough), available since 2002, administered subcutaneously at a weekly dose of 1–1.5 μg/kg in 27 patients (64.3%). Ribavirin (Copegus; Roche, Basel, Switzerland) was used at a weight-based dosage of 600–800 mg/day orally. Antiviral treatment was scheduled to last 48 weeks, independently from viral genotype and baseline viral load. No stopping rule was adopted in case of absence of complete early viral response (EVR), defined as the absence of HCV-RNA 12 weeks after the start of therapy. INF dose was reduced to 50% for leucocyte count $<1.5 \times 10^9$/l, neutrophil count $<0.75 \times 10^9$/l, platelet count $<50 \times 10^9$/l or haemoglobin level <100 g/l. INF dose was discontinued for leucocyte count $<1.0 \times 10^9$/l, neutrophil count $<0.50 \times 10^9$/l, platelet count $<25 \times 10^9$/l or haemoglobin level <85 g/l Ribavirin dose was reduced to 400–600 mg/day when haemoglobin level decreased below 100 g/l and discontinued when haemoglobin dropped below 85 g/l.

Viral methods

HCV RNA in serum was detected by means of Cobas Amplicor PCR (Roche) since 2005 and by means of real-time PCR (TaqMan; Roche) thereafter. HCV genotype was detected by means of InnoLipa genotyping kit (Imnogenetics, Zwijndrecht, Belgium) according to the manufacture's instructions.

Vitamin D supplementation

In 15 patients (35.7%), oral vitamin D3 supplementation (cholecalciferol 800 IU/day) was administered to avoid further bone loss in the presence of known pretransplant osteopenia or osteoporosis. Vitamin D3 supplementation was always initiated within the first postoperative trimester (median 21 days, range 15–39), and most patients started vitamin D3 supplementation at least 1 year before the beginning of antiviral therapy (median 425 days, range 232–879); all these patients continued vitamin D3 supplementation during the entire antiviral therapy period and none had to discontinue it temporarily due to side effects.

Vitamin D assay

For all 42 patients, serum samples, collected before starting antiviral therapy, separated and stored at -80 °C until use, were available to measure pre antiviral treatment vitamin D concentration. Circulating 25-hydroxyvitamin D levels were measured using a chemo-luminescent immunoassay implemented on a Liaison automatic analyzer (DiaSorin Inc., Stillwater, MN, USA). Data were expressed in ng/ml.

Response to antiviral therapy

The primary end-point was achievement of a SVR. The sensitivity of the PCR adopted to detect serum HCV RNA was 50 IU/ml. Secondary end-points were the achievement of EVR and of the end of treatment viral response (ETR), defined as undetectable HCV RNA (<50 IU/ml) in the serum 48 weeks after the start of treatment. Patients who did not achieve undetectable HCV RNA at the end of therapy were defined as non responders. According to an intention to treat analysis, patients who discontinued antiviral therapy for adverse events were also considered non responders.

Histology

Liver biopsies specimens were obtained before antiviral treatment. Liver biopsy was also performed on demand when clinically indicated. Specimens were evaluated by an experienced pathologist. Inflammatory activity and fibrosis were scored according to Ishak classification [22].

Statistical analysis

Statistical analysis of data was performed using the BMDP dynamic statistical software package 7.0 (Statistical Solutions, Cork, Ireland). Continuous variables were presented as median (range) and categorical variables as frequencies (%).The associations between categorical variables were performed using the Pearson chi-square test and, when appropriate, the chi-square test for linear trend; the Mann–Whitney test was used to assess differenced for continuous variables. Stepwise logistic regression analysis with a forward approach was performed in order to identify independent predictors of SVR. Time to event analysis was utilized to assess the association between vitamin D supplementation and earlier HCV RNA clearance and occurrence of SVR. The statistical analysis was performed using the Mantel–Cox test. Time to event analysis with covariates using the Cox proportional hazard model with a stepwise approach was adopted to identify which covariates were independently associated with HCV clearance and SVR.

Results

Viral response

EVR was obtained by 15 (35.7%) patients; ETR was achieved by 16 (38.1%) patients. Amongst the 26 patients who did not achieve ETR, 13 (31.0%) were truly non responders and 13 (31.0%) were drop-outs. Three patients out of 16 who obtained an ETR (18.8%) relapsed within 6 months after treatment withdrawal. Thus, SVR was observed in 13 patients (31.0%). A strict relationship was detected between obtainment of an EVR and the achievement of ETR (12/16 vs. 3/26, $P < 0.0001$) and SVR (10/13 vs. 5/29, $P = 0.0002$).

Table 2. Demographic, clinical, pathological and viral predictors of SVR in patients treated for recurrent hepatitis C. The statistical analysis was performed by means of Pearson chi-square test.

	SVR (n = 13)	No SVR (n = 29)	P
Recipient age ≤55 years	6	15	NS
Donor age ≤45 years	6	16	NS
Recipient female gender	2	10	NS
Donor female gender	3	8	NS
Body mass index ≤25 at the time of treatment	6	19	NS
Cyclosporine-based regimen	4	8	NS
Corticosteroid therapy >120 days	9	10	<0.05
Basal HCVRNA ≤400 000 IU/ml	4	9	NS
Genotype other than 1	6	4	<0.05
Days from LT to treatment >365	5	14	NS
Use of PEG-interferon α-2b	8	16	NS
Grading pre treatment ≤3	4	12	NS
Staging pre treatment ≤2	13	23	NS

NS, not significant.

Baseline predictors of SVR

Table 2 reports the main demographic, clinical, pathological and viral predictors of SVR in the studied population. The only two variables that were found to be associated with a favourable outcome of the therapy were: possessing an HCV genotype other than 1 and having been treated with steroids for more than 4 months after LT.

Baseline 25-OH vitamin D levels and SVR

Baseline 25-OH vitamin D values were stratified in three groups: severe deficiency (≤10 ng/ml) $n = 10$, deficiency (>10 and ≤20 ng/ml) $n = 20$, and near normal values (>20 ng/ml) $n = 12$. A significant linear trend ($P < 0.05$) was detected in the rate of SVR from severely deficient patients to those with near normal 25-OH vitamin D levels: 1/10 (10%), 6/20 (30%), 6/12 (50%). Step-wise logistic regression analysis with a forward approach was conducted to verify whether baseline vitamin D levels could be considered an independent predictor of SVR. The analysis considered all the variables listed in Table 2, as well as 25-OH vitamin D serum values categorized according to a cut-off value of 20 ng/ml. Infection by HCV genotype other than 1 (improvement of chi-square

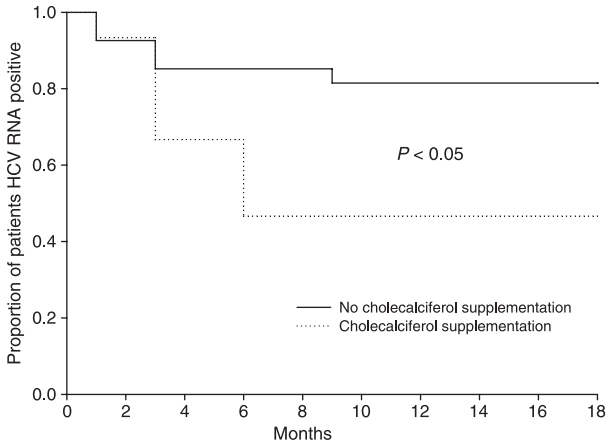

Figure 2 Time to event analysis in achieving HCV clearance after the start of antiviral treatment. Patients were divided according to cholecalciferol supplementation. The statistical analysis was performed by means of the Mantel–Cox test.

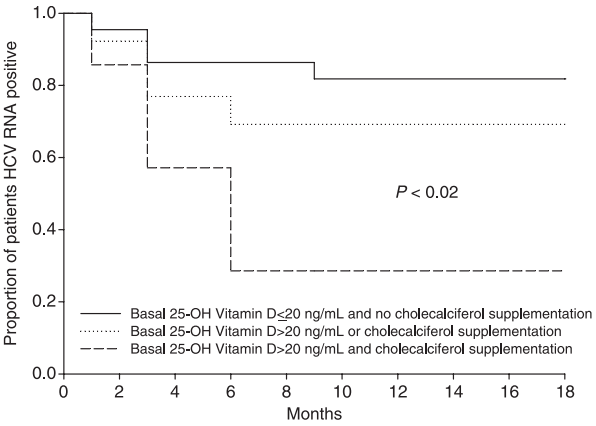

Figure 3 Time to event analysis in achieving HCV clearance after the start of antiviral treatment. Patients were divided according to baseline vitamin D status and cholecalciferol supplementation. The statistical analysis was performed by means of the Mantel–Cox test for trend.

$P < 0.05$, odds ratio 8.16, 95% confidence interval 1.39–48.0) and 25-OH vitamin D > 20 ng/ml (improvement of chi-square $P < 0.05$, odds ratio 5.31, 95% confidence interval 0.974–28.9) were found to be independent predictors of SVR.

Vitamin D supplementation and SVR

Patients who were supplemented with cholecalciferol during antiviral therapy achieved a SVR more frequently than patients who were not supplemented (8/15 vs. 5/27, $P < 0.02$). Considering the eight cholecalciferol supplemented patients who achieved a SVR, three had low basal 25-OH vitamin D levels and five had values >20 ng/ml; amongst the five not supplemented patients with SVR, four had low basal 25-OH vitamin D levels and one had basal 25-OH vitamin D levels >20 ng/ml. By time to event analysis, a significant difference was confirmed (Fig. 2) between cholecalciferol supplemented and not supplemented patients ($P < 0.05$). We therefore tried to investigate whether a synergistic effect could be detected between baseline 25-OH vitamin D levels and cholecalciferol supplementation. Patients were divided into three groups: group 1 ($n = 22$) comprising patients with basal serum 25-OH vitamin D ≤ 20 ng/ml, not supplemented with cholecalciferol, group 2 ($n = 13$) patients with basal serum 25-OH vitamin D > 20 ng/ml not supplemented with cholecalciferol ($n = 5$) or patients with basal serum 25-OH vitamin D ≤ 20 ng/ml and cholecalciferol supplemented ($n = 8$), and group 3 ($n = 7$) patients with serum 25-OH vitamin D > 20 ng/ml and cholecalciferol supplementation. A significant linear trend was observed, from group 1 to group 3, in the rate of SVR (4/22 vs. 4/13 vs. 5/7, $P < 0.02$). This finding was independent from HCV genotypes, since non significant differences were found in the prevalence of genotype 1 amongst the three groups: 15/22 (68.2%) in group 1, 12/13 (92.3%) in group 2, 5/7 (71.4%) in group 3 ($P > 0.40$). Time to event analysis confirmed the differences amongst the three abovementioned groups in terms of time to become HCV RNA negative ($P < 0.02$) (Fig. 3). To assess whether the relationship between baseline 25-OH vitamin D and cholecalciferol supplementation could indeed be considered an independent predictor of SVR, a Cox proportional hazard model was applied. The analysis considered all the variables presented in Table 2 as well as the combination of baseline serum vitamin D and cholecalciferol supplementation. Having received cholecalciferol supplementation in the presence of a normal or near normal baseline serum vitamin D concentration (improvement of chi-square $P < 0.05$, odds ratio 2.22, 95% confidence interval 1.167–4.223) and possessing a genotype other than 1 (improvement of chi-square $P < 0.05$, odds ratio 3.383, 95% confidence interval 1.131–10.116) were the only variables independently associated to SVR.

Discussion

Vitamin D is either synthesized in the skin following exposure to ultraviolet B radiation or ingested with the diet; in the organism it is then stored in fat cells. To produce the biologically active form, vitamin D is subjected to two hydroxylations, the first in the liver, originating 25-OH vitamin D, and the second in the kidney, which produces 1,25-OH vitamin D. The active form of vitamin D enters the cells and binds to its receptor; the complex

vitamin D-vitamin D receptor then heterodimerizes with the retinoid X receptor and binds to vitamin D response elements in the promoter of target genes, thereby affecting their transcription [23]. The vitamin D receptor is expressed in several types of cells, accounting for by the pleyotropic actions ascribed to vitamin D [19]. Besides the classical actions related to calcium homeostasis and bone metabolism, vitamin D has emerged as a key regulator of innate immunity in humans [24,25]. In particular a relationship has been suggested between vitamin D status and susceptibility to infectious diseases. In man, the risk of being infected with *Mycobacterium tuberculosis* has been shown to be associated with vitamin D deficiency [26–29]. Some experimental data support this assumption: (i) macrophages express vitamin D receptor [23], (ii) when infected from by *Mycobacterium tuberculosis*, macrophages engage a toll like receptor response that enhances the vitamin D receptor expression [30], (iii) in a mirror way, vitamin D robustly stimulates the expression of pattern of recognition receptors such as NOD2 and CARD15 [31]. Furthermore, a relationship has been detected between vitamin D deficiency and the risk of developing upper respiratory tract viral infections [32], and a recent placebo-control trial demonstrated a reduction in the rate of influenza infection in subjects treated with 2000 IU/day of Vitamin D [33].

In this study, for the first time, vitamin D status at the time of starting antiviral therapy was found to be associated with the achievement of SVR following treatment of RHC. The association was strictly related to the degree of vitamin D deficiency: patients with severe vitamin D deficiency (≤10 ng/ml) almost never achieved SVR, whilst those with near normal or normal vitamin D (>20 ng/ml) prior to starting antiviral treatment obtained a SVR rate in about half the cases. These results deserve several comments. First, they confirm that LT recipients are frequently severely vitamin D deficient. Furthermore, 31/42 patients (74%) remained vitamin D deficient (≤20 ng/ml) despite vitamin D supplementation. In our series, oral cholecalciferol was administered at the conventional daily dose of 800 IU to correct osteopenia and osteoporosis: in fact, it was initiated independently from vitamin D serum level and not to correct vitamin D deficiency. Therefore, the observation that approximately 50% of supplemented patients were still vitamin D deficient at the start of antiviral therapy was not entirely unexpected. Whether 1,25-OH rather than 25-OH vitamin D assay should be performed in this setting remains to be ascertained; nevertheless it must be pointed out that 25-OH is the major circulating form of vitamin D and is used as an indicator of vitamin D status [34].

To our knowledge, there is only one report in the literature dealing with the association between vitamin D status and outcome of antiviral therapy for chronic HCV infection. Petta *et al.* [21], analyzing retrospectively a cohort of 167 immune competent patients treated with Peg-interferon and ribavirin for chronic hepatitis C, detected an association between lower vitamin D levels and failure to achieve SVR. Our results give further support to these data in a different setting, although some differences between the two studies need to be pointed out. In fact, LT patients are more severely vitamin D deficient than immune competent patients with chronic HCV infection. Accordingly, our chosen vitamin D cut-offs were in a lower range in comparison to the average levels reported by Petta *et al.* [21]. In view of the high prevalence of vitamin D deficiency we observed, it is conceivable to speculate that vitamin D may play a more relevant role in modulating viral clearance in RHC rather than amongst immune-competent patients. Taken altogether, these data suggest a causative role for vitamin D in influencing the rate of HCV clearance following antiviral treatment. In fact, if vitamin D concentration were considered just a surrogate marker of other, unknown unfavourable conditions able to influence SVR, no effect at all might derive from vitamin D supplementation on the outcome of antiviral treatment.

Several predictors of SVR have been proposed in patients affected by RHC. Whilst some of them are specific to the LT setting, others are shared by immune-competent patients affected by chronic hepatitis C. Type of immune-suppression [12,13,16–18], length of steroid therapy, timing from LT to starting antiviral treatment [35] are peculiar to LT; gender, age, body mass index, HCV genotype, viral load, type of INF, and degree of fibrosis are known predictors for both immune competent and immune-suppressed patients. In the present study, HCV genotype was confirmed as an important predictor of SVR in patients with RHC [16,17], in association with vitamin D. Other variables were not confirmed as predictors, except, at univariate analysis, the length of steroid treatment following LT. This finding confirms recent data suggesting that longer steroid treatment, in combination with azathioprine and tacrolimus versus tacrolimus mono-therapy, favours a better outcome of RHC after LT [36].

Should vitamin D supplementation be proposed to all LT patients before starting antiviral treatment for RHC? We cannot deny the limits of a retrospective study in a setting, that of LT, where many confounding variables may interact. For a definitive answer, a proper and larger prospective, placebo-controlled, randomized trial should be designed. Nevertheless, such a study would be hampered by the fact that the majority of LT patients are severely vitamin D deficient: in fact, it might be considered nonethical to avoid vitamin D supplementation to

patients in the control arm, unless only patients with normal vitamin D concentration are included. Finally, vitamin D supplementation might reduce the probability of rejection episodes. In fact, higher pretransplant vitamin D levels and post-transplant vitamin D supplementation were found to be associated with a lower probability of rejection [20]. This effect may be of particular value considering that antiviral therapy may enhance the risk of acute cellular rejection in patients with RHC [15].

In conclusion, vitamin D deficiency is a frequent finding in the patients transplanted for HCV related end stage liver disease and predicts an unfavourable response to antiviral treatment of RHC. Vitamin D supplementation improves the probability of achieving a SVR following antiviral treatment.

Authorship

DB designed the research. DB, AC and EF performed the research. EF, EF and SB collected the data. CP, SC, EF and RM contributed important reagents. CF analyzed the data. DB, CF, MP and PT wrote the paper.

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