Severe Vitamin D Deficiency Increases Mortality Among Patients With Liver Cirrhosis Regardless of the Presence of HCC

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Abstract. *Background: The aim of this study was to investigate the association between vitamin D deficiency (<10 mg/ml) and mortality in patients with and without hepatocellular carcinoma (HCC) in a cohort of patients with liver cirrhosis. Materials and Methods: A prospective study was conducted among 345 patients with liver cirrhosis. Results: At enrolment, 46 (13.3%) patients had HCC. Severe vitamin D deficiency was associated with mortality ($p<0.01$). At the survival analysis, alpha-fetoprotein >10 ng/ml ($p=0.003$), vitamin D deficiency ($p<0.001$), a Model for End-Stage Liver Disease score ≥ 15 ($p<0.001$), Child-Pugh class B and C (versus A) ($p<0.001$) and the presence of active HCC ($p<0.001$) were strongly associated with death. At the multivariate Cox regression analysis, only Child-Pugh class B and C (versus A) and vitamin D deficiency were found to be significantly associated with death during the follow-up period ($p<0.001$ and $p=0.006$, respectively). Conclusion: Vitamin D deficiency is common in patients with HCC, it is associated with active HCC and it negatively affects the overall survival of patients with cirrhosis.*

Hepatocellular carcinoma (HCC) is one of the major complications of liver cirrhosis. It is the sixth most-diagnosed

malignancy worldwide (1) and its incidence among patients with liver cirrhosis is estimated to be 1-8% per year (2). Moreover, HCC is often diagnosed in an advanced stage and therefore treatment options are often limited (3). In patients with cirrhosis, several factors are associated with a higher risk of developing HCC, for instance, co-infection with hepatitis C virus (HCV)/human immunodeficiency virus (HIV)/hepatitis B virus (HBV), obesity, steatosis, diabetes mellitus, and alcohol abuse (4-7). The interest in new factors potentially involved in the occurrence of HCC increased recently due to evidence that some patients with chronic HCV infection may show a *de novo* occurrence of HCC during or after treatment with new directly acting antiviral agents, despite their high efficacy in curing viral infection (8-11). In fact, new anti-viral treatments for HCV are able to reach very high rates of viral eradication with scarce side-effects differently from previous antiviral treatments for HCV (interferon era) (12-14).

Vitamin D exerts several biological effects other than the well-known regulation of calcium and phosphorus metabolism: it has a role in activating innate and adaptive immunity (15, 16) thus having a potential role in preventing infection, and it is also able to influence neo-angiogenesis (17,18) and pathways of apoptosis (19, 20). Accumulating evidence has shown that vitamin D deficiency is associated with a higher risk of developing different types of cancer (21-23) and it may, thus, have a role in the diagnosis of HCC, together with other markers already known to be useful in the diagnosis of such neoplasia [*e.g.* alpha-fetoprotein (AFP), protein induced by vitamin K absence-II and their combination] (24). However, little is known about the relationship of vitamin D deficiency with HCC risk, and mortality among patients with liver cirrhosis. In a European nested-control study, low levels of serum vitamin D pre-diagnosis were associated with an increased risk of HCC development regardless of pre-existing liver disease (25), probably augmenting the mortality rate in such patients.

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The aim of our study was to investigate the association between vitamin D deficiency and mortality in patients with and without HCC in a cohort of patients with liver cirrhosis.

Materials and Methods

Study design. Inpatients and outpatients referring to the Department of Clinical Medicine and Surgery – Section of Infectious Diseases of the Federico II University Hospital of Naples were prospectively enrolled from May 1st 2013 to July 31st 2016. Inclusion criteria were: age ≥ 18 years and a diagnosis of liver cirrhosis (regardless of etiology). Exclusion criteria were: i) oral supplementation with cholecalciferol (vitamin D) or derivatives in the previous 12 months, ii) refusal of consent. Patients enrolled underwent follow-up visits every 6 months until April 30st 2017. Follow-up visits were performed before schedule whenever the patients required medical assistance, such as for the occurrence of a cirrhosis-related complication.

Diagnosis of cirrhosis was made by clinical criteria, histological findings, or a liver stiffness ≥ 13 kPa at FibroScan® (26). Clinical criteria for the diagnosis of cirrhosis included both laboratory (hypoalbuminemia, thrombocytopenia, low pseudocholinesterase level, decrease in prothrombin activity) and ultrasonographic findings, as well as the occurrence of cirrhosis-related complications, namely jaundice, ascites and porto-systemic encephalopathy as reported elsewhere (27-29). Demographic and clinical data of all the enrolled patients were collected at baseline, together with blood samples for biochemistry and complete blood counts. One blood sample was collected from each patient for centralized determination of serum 25-OH-vitamin D. Vitamin D insufficiency was defined as serum 25-OH-vitamin D concentrations between 20 and 30 ng/ml, while deficiency and severe deficiency were defined as serum concentrations of 10-20 ng/ml and ≤ 10 ng/ml, respectively (30).

Finally, the presence of HCC was investigated in each patient at enrollment. Patients with a diagnosis of HCC at enrollment were divided into those with active HCC and those with a previous diagnosis of HCC. Diagnosis of HCC was made by radiological, histological or cytological criteria, in accordance with the American Association for the Study of the Liver (AASLD) HCC guidelines (31). Among patients with active HCC at enrollment, both the presence of portal vein invasion and the presence of metastasis were investigated. At each follow-up visit, clinical information of the enrolled patients was collected. Moreover, all patients underwent ultrasonography every 6 months and, in cases of detection of a suspicious or typical lesion, they were managed according to AASLD guidelines (31) and the date of the diagnosis was recorded. Finally, last visit to our Department was evaluated to perform survival analysis. Missing data regarding the occurrence of HCC or serum 25-OH-vitamin D concentrations, as well as those lost to follow-up and those receiving vitamin D supplementation during the observational period, were considered exclusion criteria during the study.

Outcomes of the study and sample size. The primary outcome of the study was to estimate the incidence of HCC among patients with liver cirrhosis and severe deficiency of vitamin D.

Secondary outcomes were to: analyze the difference in 25-OH-vitamin D concentrations between patients with and those without active HCC; analyze the risk factors for new-onset HCC among patients with liver cirrhosis; and estimate the mortality rate among patients with severe vitamin D deficiency and HCC.

Given an HCC incidence rate of 6% of among patients with liver cirrhosis, we estimated that to show an incidence rate at least 1.5-fold higher among patients with severe vitamin D deficiency and liver cirrhosis the calculated sample size was 72 (α -error=0.05; power=0.8).

Statistical analysis. Kolmogorov–Smirnov test was applied to quantitative variables to check for Gaussian distribution. Data are given as mean $\pm$ standard deviation or as median and interquartile range (IQR) in the case of Gaussian and non-Gaussian distribution, respectively. For comparisons of categorical variables, the chi-squared test (or Fisher's exact test if appropriate) was used, while the *t*-test or the Mann–Whitney *U*-test were used for comparisons of continuous variables for Gaussian-distributed or non-Gaussian-distributed data, respectively. Logistic regression model was used to estimate the odds ratio (OR), while Poisson regression with robust variance estimator was used to estimate the incidence risk ratio (RR). The variables that were significantly associated at univariate analysis were included in the multivariate model analysis. The Kaplan–Meier model was used for survival analysis. The effect of single variables on survival was assessed using the log-rank test. The variables that showed a significant association in this test were included in the Cox regression multivariate test. For all tests, a two-sided *p*-value of less than .05 was considered statistically significant. Statistical analysis was carried out using the Statistical Package for the Social Sciences version 2.0 (IBM, Armonk, NY, USA).

Ethical statement. The present prospective study was approved by the Ethical Committee of the Federico II University of Naples (Prot. No. 128/12) and was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki, Sixth Version) for experiments involving humans. Written informed consent was obtained from each patient included in the study.

Results

Three-hundred and forty-five patients were enrolled, none of them was excluded during the study due to missing data on oral vitamin D supplementation during the study. Clinical features of enrolled patients are shown in Table I. At enrollment, 46/345 (13.3%) patients had a diagnosis of HCC and, 23/46 of these (50%) had active HCC, while the other half had a history of successfully treated HCC. Among patients with active HCC at enrollment, 9/23 (39.1%) had local extension of the neoplasia to the portal vein, while 8/23 (34.8%) had extrahepatic metastasis. Table I also shows the clinical parameters of patients with and without active HCC. Patients with Child-Pugh class B or C cirrhosis showed an increased risk of HCC compared with those with class A, as well as those with serum 25-OH-vitamin D concentrations < 10 ng/ml. Moreover, the median serum vitamin D concentration was significantly lower among patients with HCC compared to those without. Finally, patients with a diagnosis of diabetes mellitus showed an increased risk of HCC, even though such a result was of borderline statistical significance (OR=2.18, 95% CI=0.91-5.27; $p=0.082$).

At baseline, the median serum 25-OH-vitamin D concentration was 15 ng/ml (IQR=8-24 ng/ml). Few patients

Table I. Clinical and laboratory parameters of patients with and without active hepatocellular carcinoma (HCC).

Parameter	All patients (N=345)	Active HCC (n=23)	No HCC (n=322)	OR (95% CI)	p-Value
Median age (IQR), years	68 (61-74)	71 (65-78)	68 (61-74)	0.96 (0.92-1.01)	0.130
Male, n (%)	268 (77.7)	17 (73.9)	184 (57.1)	2.12 (0.82-5.53)	0.743
Child-Pugh class, n (%)					
A	268 (77.7)	10 (43.5)	258 (8.1)	0.19 (0.08-0.45)	<0.001
B	56 (16.2)	9 (39.1)	47 (14.6)	3.76 (1.54-9.18)	0.004
C	21 (6.1)	4 (17.4)	17 (5.3)	3.78 (1.16-12.34)	0.028
Etiology of cirrhosis, n (%)					
HCV	335 (97.1)	23 (100)	312 (96.9)	#	#
HBV	2 (.6)	0 (0)	2 (.6)	#	#
Alcohol abuse	4 (1.2)	0 (0)	4 (1.2)	#	#
Cryptogenic	4 (1.2)	0 (0)	4 (1.2)	#	#
Diabetes, n (%)	136 (39.4)	13 (56.5)	123 (38.2)	2.18 (.91-5.27)	0.082
Vitamin D <10 ng/ml, n (%)*	111 (32.2)	12 (52.2)	99 (3.7)	2.46 (1.05-5.76)	0.039
Median glucose (IQR), mg/dl	101 (88-120)	101 (89-135)	101 (87-120)		0.681
Median albumin (IQR), g/dl	3.7 (3.4-4.1)	3.4 (2.8-3.7)	3.8 (3.4-4.1)		0.146
Median creatinine (IQR), mg/dl	1.0 (1.0-1.0)	1.5 (1.0-1.5)	1.0 (1.0-1.0)		0.351
Median AST (IQR), IU/ml	48 (30-83)	75 (36-168)	47 (29-81)		0.013
Median ALT (IQR), IU/ml	43 (22-72)	43 (28-98)	43 (21-71)		0.438
Median total bilirubin (IQR), mg/dl	1.03 (.7-1.6)	1.4 (.9-2.3)	1.0 (.7-1.5)		0.281
Median direct bilirubin (IQR), mg/dl	0.4 (.2-.6)	0.6 (.3-1.8)	0.4 (.2-.6)		0.107
Median PCHE (IQR), mg/dl	3,728 (2,133-5,768)	2,151 (1,414-3,338)	4,069 (2,366-6,063)		0.160
Median WBCs (IQR), $\times 10^3/\text{mm}^3$	5.115 (3.852-6.622)	5.200 (1.230-8.510)	5.110 (3.845-6.500)		0.560
Median RBCs (IQR), $\times 10^6/\text{mm}^3$	4.26 (3.77-4.73)	4.03 (3.36-4.23)	4.28 (3.78-4.75)		0.016
Median Hb (IQR), g/dl	13.1 (11.3-14.6)	12.5 (9.7-13.1)	13.2 (11.4-14.6)		0.002
Median PLTs (IQR), $\times 10^3/\text{mm}^3$	109 (74-156)	102 (62-156)	109 (75-156)		0.627
Median INR	1.16 (1.06-1.28)	1.23 (1.17-1.38)	1.15 (1.06-1.27)		0.195
Median AFP (IQR), ng/ml	7.0 (3.8-13.5)	211.0 (26.2-968.7)	7.0 (3-12.0)		<0.001
Median vitamin D (IQR), ng/ml*	15.0 (8.0-24.0)	9.0 (4.9-17.0)	15.0 (8.0-24.0)		0.010

OR: Odds ratio; CI: confidence interval; AST: aspartate-transaminases; ALT: alanine-transaminases; ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase; PCHE: pseudocholinesterase; WBCs: white blood cells; RBCs: red blood cells; Hb: hemoglobin; PLTs: platelets; INR: international normalized ratio; AFP: α -fetoprotein. *25-OH-Vitamin D. #Not calculable, the etiology of cirrhosis was HCV for all patients with active HCC.

(52/345, 15%) were found to have a sufficient level of vitamin D (Figure 1).

During the follow-up (median=9 months, IQR=6-16 months), among the 299 patients without HCC at baseline, a total of 13 patients (4.4%) had a diagnosis of *de novo* HCC, while out of the whole cohort, 54 (15.7%) died. Among patients with severe vitamin D deficiency, 6/111 (5.4%) developed *de novo* HCC during the follow-up, while 7/234 (2.9%) patients with serum 25-OH-vitamin D concentrations >10 ng/ml had a new diagnosis of HCC ($p=0.162$). The person-per-year incidence was 0.08 and 0.03 among patients with and without severe vitamin D deficiency, respectively. The RR of HCC among patients with severe vitamin D deficiency was 2.19 (95% CI=0.71-6.73, $p=0.171$).

AFP>10 ng/ml was associated with HCC onset during the first 12 months of follow-up (RR=1.18; 95% CI=1.16-89.91, $p=0.036$) (Table II). In the survival analysis, AFP>10 ng/ml ($p=0.003$), 25-OH-vitamin D <10 ng/ml ($p<0.001$) (Figure 2), a Model for End-Stage Liver Disease score ≥ 15 ($p<0.001$), Child-Pugh class B and C (*versus* A) ($p<0.001$)

and the presence of active HCC ($p<0.001$) were strongly associated with death.

At multivariate Cox regression analysis, which included all the covariates significantly associated with death by log-rank test, only Child-Pugh class B and C (*versus* A) and 25-OH-vitamin D <10 ng/ml were found to be significantly associated with death during the follow-up period (HR=6.27; 95% CI=2.09-18.77; $p<0.001$ and HR=4.33; 95% CI=1.54-12.20; $p=0.006$, respectively) (Table III).

Discussion

In our cohort of patients with liver cirrhosis, we found a high prevalence (64%) of vitamin D deficiency. This is probably due to the impairment of several mechanisms implicated in vitamin D absorption in advanced liver disease, such as activation and hydrolyzation of 25-OH-cholecalciferol or malabsorption due to portal hypertension. It is noteworthy that vitamin D deficiency is associated with a high rate of HCC at presentation. This finding can be explained by the known

Figure 1. Rates of vitamin D insufficiency (25-OH-vitamin D=20-30 ng/ml), deficiency (10-<20 ng/ml) and severe deficiency (<10 ng/ml) in study patients (N=345).

Figure 2. Cumulative survival in patients with and without severe deficiency of vitamin D (25-OH-vitamin D <10 ng/ml).

Table II. Univariate analysis of factors associated with hepatocellular carcinoma onset in the first 12 months of follow-up.

Factor	RR	95% CI	p-Value
AFP >10 ng/ml	1.18	1.16-89.21	0.036
25-OH-Vitamin D <10 ng/ml	2.19	0.71-6.73	0.171
Male sex	1.19	0.38-3.72	0.769
MELD >15	3.41	0.87-13.37	0.078
Decompensated cirrhosis*	1.49	0.39-5.65	0.555
Serum ferritin concentration	1.00	0.99-1.00	0.990

RR: Incidence risk ratio; CI: confidence interval; AFP: α -fetoprotein; MELD: Model for End-Stage Liver Disease score. *Child-Pugh class B or C.

Table III. Cox regression analysis for mortality.

Factor	HR	95% CI	p-Value
AFP >10 ng/ml	2.03	0.82-5.07	0.127
25-OH-Vitamin D <10 ng/ml	4.33	1.54-12.20	0.006
MELD $\geq$ 15	1.34	0.54-3.35	0.524
Child-Pugh B or C classification	6.27	2.09-18.77	<0.001
Active HCC	2.23	0.88-5.66	0.093

HR: Hazard ratio; CI: confidence interval; AFP: α -fetoprotein; MELD: model for end-stage liver disease score; HCC: hepatocellular carcinoma.

properties of vitamin D, which has direct effects on cell differentiation and proliferation (32-34). We showed that HCC incidence was not significantly increased among patients with severe vitamin D deficiency. However, a significantly higher mortality was found in patients with liver cirrhosis and severe vitamin D deficiency compared with patients with serum 25-OH-vitamin D concentrations >10 ng/ml.

We underline that, even though it is known that vitamin D levels are lower in patients with advanced-stage liver disease according to Child-Pugh classification (35), in our cohort, severe deficiency of vitamin D remained associated with poor survival independently of Child-Pugh class (B or C versus A) at multivariate analysis. However, this role might be totally independent of liver disease. In fact, vitamin D deficiency is found in many other diseases, for example in cardiovascular diseases; in this setting a large trial is currently ongoing (VITAL trial). This trial was designed to investigate the role of both vitamin D and omega-3 fatty acid supplementation in the reduction of heart diseases and stroke incidence in people without a prior history of these illnesses (36, 37). Results from these studies raise the hypothesis that the strong association between vitamin D deficiency and

mortality is not only related to advanced liver disease. For instance, we showed that infection significantly reduces survival in patients with cirrhosis (38) and that vitamin D deficiency is associated with infection in such patients (35). Moreover, it is noteworthy that vitamin D deficiency affects the survival rate in patients with liver cirrhosis. In fact, there are different studies demonstrating this association but, to our knowledge, this is the first study demonstrating this association irrespective of the presence of HCC, and in a sample of patients with low prevalence of post-alcoholic cirrhosis. Notably, in the abovementioned studies, the prevalence of patients with post-alcoholic liver cirrhosis was remarkably higher than in our study (from 36% to 61% in previous studies *versus* 1.2% in our study). Furthermore, it is conceivable that alcohol abuse may *per se* worsen prognosis of liver cirrhosis and affect vitamin D level.

Our study had certain limitations. Firstly, it was not powered enough to highlight possible correlation between severe vitamin D deficiency and active HCC. In fact, even though the prevalence of severe vitamin D deficiency was significantly higher among patients with active HCC, we cannot state that a cause-effect link is present. Moreover, as this was not an interventional study, we cannot state whether exogenous vitamin D supplementation may have a role in reducing mortality in patients with liver cirrhosis. In this regard, our study paves the way to further interventional research.

In conclusion, we demonstrated that in patients with liver cirrhosis, vitamin D deficiency was associated with poor survival irrespective of the presence of HCC. Therefore, randomized controlled trials aiming to confirm this association and demonstrate the potential role of vitamin D supplementation in improving survival rates are needed.

Conflicts of Interests

I.G. acted as a consultant for AbbVie and MSD and received a grant from Gilead Sciences (in the framework of Fellowship program). The other Authors have no conflicts of interest to declare. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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