

RESEARCH ARTICLE

Low 25-Hydroxyvitamin D Levels Are Associated with Infections and Mortality in Patients with Cirrhosis

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Abstract

Background & Aims

Vitamin D, best known to regulate bone mineralization, has numerous additional roles including regulation inflammatory pathways. Recently, an increased incidence of 25-hydroxyvitamin D₃ (25(OH)D₃) deficiency has been found in subjects suffering from liver diseases. We here investigated if low vitamin D levels might be associated with prognosis, inflammation and infectious complications in patients with cirrhosis.

Methods

We performed a prospective cohort study investigating the relation between 25(OH)D₃ levels and stages of cirrhosis, mortality and complications of cirrhosis, including infections.

Results

251 patients with cirrhosis were enrolled into the present prospective cohort study. 25(OH)D₃ levels were quantified by radioimmunoassay from serum samples obtained at study inclusion. The mean follow-up time was 411 ± 397 days with a range of 1-1382 days. 30 (12.0%) patients underwent liver transplantation and 85 (33.8%) individuals died within the study. The mean serum 25(OH)D₃ concentration was 8.93 ± 7.1 ng/ml with a range of 1.0 to 46.0 ng/ml. 25(OH)D₃ levels differed significantly between Child Pugh scores and showed a negative correlation with the model of end stage liver disease (MELD) score. Patients with decompensated cirrhosis and infectious complications, had significantly lower 25(OH)D₃ levels compared to subjects without complications. Low 25(OH)D₃ was associated with mortality in uni- as well as multivariate Cox regression models.

Conclusions

25(OH)D₃ deficiency is associated with advanced liver disease and low 25(OH)D₃ levels are an indicator for a poor outcome and are associated with infectious complications.

Introduction

Cirrhosis is a consequence of chronic liver diseases, characterized by liver injury, chronic inflammation and finally by progressive substitution of liver parenchyma by scar tissue. Cirrhosis results in progressive liver failure and is accompanied by various complications such as portal hypertension, ascites, gastrointestinal bleeding or hepatocellular carcinoma (HCC) [1]. It accounts for about 2% of all deaths in Europe (170.000/year) with increasing mortality rates in several countries [2]. Alcohol abuse and infections with hepatotropic viruses, especially hepatitis C virus (HCV) infection, have been the predominant risk factors for cirrhosis in Western countries within the last decades. However, the prevalence of non-alcoholic fatty liver disease (NAFLD) has risen dramatically, affecting up to 40% of all Europeans and more than 50% of patients suffering of type 2 diabetes within the last years [3]. Patients with NAFLD are at risk to develop non-alcoholic steatohepatitis (NASH) which is supposed to become the major cause of cirrhosis within the following decades [4]. The prognosis of cirrhotic individuals is determined by the occurrence of hepatic complications [3]. Patients with compensated cirrhosis without clinically evident portal hypertension have a relatively favorable prognosis. On the other hand, patients with decompensated disease, who suffer from ascites as well as variceal bleeding have one year mortality rates of more than 50% [3]. Commonly used predictors of prognosis are the Child-Pugh-Score [4] and the model for end stage liver disease (MELD) [5].

Vitamin D is best known for its function in calcium homeostasis and bone mineralization [6]. However, it has numerous additional roles such as regulation of proliferation, apoptosis, differentiation and inflammation [7,8]. Vitamin D generation is a multi-step process involving the skin, the liver and the kidneys. Cholecalciferol is hydroxylated to the bioactive 25-hydroxyvitamin D₃ (25(OH)D₃) in the liver and is bound to the vitamin-D-binding-protein (DBP) [8]. 1 α -hydroxylase converts 25(OH)D₃ to 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)D₃) mainly in the kidneys. 1,25(OH)D₃, known as calcitriol, is the most bioactive form [8]. Recently, an increased incidence of 25(OH)D₃ deficiency has been found in individuals suffering from liver diseases and the severity of 25(OH)D₃ deficiency in the patients correlated with the severity of liver dysfunction [9,10,11]. Liver disease is accompanied by activation of the innate immune system and vitamin D levels inversely correlate with the expression of toll like receptors (TLRs) in monocytes, indicating an inverse correlation between vitamin D levels and systemic inflammation [9]. Strong inflammatory conditions displayed by high levels of C-reactive protein (CRP) or soluble CD163 (sCD163) are associated with an unfavorable prognosis in patients with cirrhosis [12,13]. Observations in cirrhotic patients showed a poor prognosis in individuals with low 25(OH)D₃ levels [14,15]. Given the fact that cirrhotic patients presenting with infections or elevated inflammatory conditions indicated by high levels of CRP or sCD163, have an unfavorable prognosis [12,13,16] and that expression of TLRs inversely correlate with vitamin D levels [9], we hypothesized that low vitamin D levels might be associated with inflammatory responses and infectious complications in cirrhotic patients. Therefore, we performed a prospective cohort study investigating the relation of 25(OH)D₃ levels with stages of cirrhosis and mortality as well as the association of 25(OH)D₃ concentrations and complications of cirrhosis, including infectious complications.

Patients and Methods

Selection of patients

Between May 2009 and June 2011 patients with cirrhosis presenting at the Department of Internal Medicine 1 of the Frankfurt University Hospital were consecutively enrolled into the

present prospective cohort study. All patients gave their written informed consent prior to study inclusion. The study population has been described before [13].

The inclusion criteria were cirrhosis assessed by liver histopathological examination or pathognomonic results in ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI). Exclusion criteria were patients below 18 years of age, a history of cancer other than hepatocellular carcinoma within the last five years or a history of solid organ transplantation. Furthermore, patients with cholestatic liver diseases, namely primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) were excluded from the analysis. Patients who were eligible for liver transplantation were listed for liver transplantation. Organ allocation was performed by Eurotransplant according to Eurotransplant and German guidelines. Patients were included in the study from the day of written informed consent and were followed-up until death, liver transplantation or last contact. Patients who underwent liver transplantation were excluded from further analysis from the day of transplantation. In patients who presented with clinical signs of infections (fever, chills, coughing or dysuria) or showed elevated CRP or leukocyte values in the blood tests, urinalysis and x-ray of the chest were performed. If urinalysis showed presence of leukocytes, urine cultures were initiated. In patients who had clinical or radiological signs of lung infections sputum cultures were done. In severely sick patients with pneumonia bronchoalveolar lavage was performed to gain material for cultures. If diarrhea was reported in patients with suspected infection, stool cultures were initiated. If ascites was present paracentesis was mandatory to rule out SBP.

Child-Pugh [3] and MELD [4] and scores were assessed by clinical examination, laboratory parameters and results of abdominal ultrasound examination, CT or MRI at the time of inclusion in the study. Diagnosis of infection was based on clinical presentation and laboratory results. Diagnosis and treatment of ascites with or without spontaneous bacterial peritonitis were performed according to EASL guidelines [17].

The study was performed in accordance with the 1975 Declaration of Helsinki and the REMARK guidelines [18] for prospective biomarker studies. The study was approved by the institutional review board of the Frankfurt University Hospital.

Blood sampling

Blood sampling and storage of samples was performed as described before [13]. Routine laboratory parameters were determined at the Central Laboratory of the Frankfurt University Hospital.

Vitamin D measurements

25(OH)D₃ levels were quantified by radioimmunoassay (I125 Radioimmunoassay IA Kit; DiaSorin, Stillwater, MN) on a Multi Crystal LB2111 Gamma Counter (Berthold, Bad Wildbad, Germany) from serum samples obtained at the day of study inclusion as described recently [19]. Samples were blinded for the person who performed measurements, and all measurements were determined at the same day from prospectively collected and stored serum samples. Serum 25(OH)D₃ concentrations <10 ng/ml were defined as severe vitamin D deficiency; levels from 10 to 20 ng/ml were considered as insufficiency and serum levels >20 ng/ml were considered normal. Serum soluble CD163 (sCD163) concentrations were assessed with the Macro163 sandwich enzyme-linked immunosorbent assay (ELISA) (Trillium Diagnostics, Bangor, ME) according to the recommendation of the manufacturer as described previously [13]. Samples were measured in duplicates on a Tecan SLT Rainbow plate reader (Tecan, Mänedorf, Switzerland).

Statistical analysis

This study was designed as a prospective cohort study. Patients were included on the day of presentation after giving written informed consent. They were followed until death or last contact. The primary end point was overall survival. Predictors of survival were determined using a univariate Cox regression hazard model. Death was recorded as event. For assessment of independent predictors of survival a multivariate Cox regression hazard model with forward stepwise (likelihood ratio) entry was used. Survival curves with the estimated hazards were calculated with the Cox regression model. Statistical analyses were performed with SPSS (Version 22.0, IBM, New York, USA) and BiAS (Version 10.03, Epsilon-Verlag, Darmstadt, Germany). Continuous variables are shown as means \pm standard deviation and categorical variables are reported as frequencies and percentages. Differences in the serum biomarker values between different patient cohorts were determined using the nonparametric Wilcoxon-Mann-Whitney and Kruskal-Wallis tests. For sub-analysis of a statistically significant Kruskal-Wallis test, the Bonferroni correction was used. *P* values < 0.05 were considered to be significant. In the box plots the vertical lines indicate the range, the horizontal boundaries of the boxes represent the first and third quartile. The correlation coefficient *r* between different parameters was calculated by using the Spearman correlation.

Results

251 patients with cirrhosis were included in the present study. Patients' characteristics are summarized in [Table 1](#). In the predominant number of cases cirrhosis was a result of alcohol abuse, hepatitis B virus (HBV) or HCV infection. 46 patients (18.3%) concurrently suffered from HCC. 192 patients (76.5%) showed complications of cirrhosis (ascites, spontaneous bacterial peritonitis (SBP), gastrointestinal bleeding, hepatorenal syndrome (HRS) or hepatic encephalopathy) at the day of inclusion in the study, whereas 59 patients (23.5%) had compensated disease. The mean follow-up time was 411 \pm 397 days with a range of 1–1382 days. 30 patients (12.0%) underwent liver transplantation within the observation time after study inclusion and were excluded from further analysis from the day of transplantation. 85 individuals (33.8%) died within the study. The main reasons of death were liver failure, sepsis with concomitant multi organ failure, and variceal bleeding.

25(OH)D₃ levels among different etiologies of chronic liver disease

The mean serum 25(OH)D₃ concentration was 8.9 \pm 7.1 ng/ml with a range of 1.0 to 46.0 ng/ml. 173 patients (68.9%) had very low 25(OH)D₃ levels (< 10 ng/ml), 59 patients (23.5%) had low levels (10–20 ng/ml) and 19 (7.6%) patients had normal 25(OH)D₃ levels (> 20 ng/ml).

44 patients (17.5%) received oral vitamin D supplementation at the day of study enrollment. Of the 44 patients, 30 patients received 1000 IU or less 25(OH)D₃ per day and six subjects took more than 1000 IU 25(OH)D₃ daily. Eight individuals were supplemented with 250 μ g 1 α ,25(OH)₂D₃ per day. One of the patients taking 250 μ g 1 α ,25(OH)₂D₃ per day was also on 25(OH)D₃ supplementation (1000 IU per day). The mean serum 25(OH)D₃ concentration in subjects with vitamin D supplementation was significantly higher (12.4 \pm 9.6 ng/ml) compared to individuals without supplementation (8.2 \pm 6.2 ng/ml) (*P* = 0.003). As the main etiologies of cirrhosis were alcohol abuse and infections with HBV or HCV, 25(OH)D₃ levels were compared between patients with and without alcoholic liver disease, HBV or HCV infections. 25(OH)D₃ levels did not significantly differ between patients with or without HBV or HCV infection (*P* = 0.117 and *P* = 0.092, respectively). However, patients with alcoholic liver cirrhosis had significantly lower serum 25(OH)D₃ concentrations compared to individuals with other causes of cirrhosis (*P* = 0.001) ([Fig 1A](#)). There was no significant difference in 25(OH)D₃ levels

Table 1. Patient characteristics.

Parameter	Patients
Epidemiology	
Patients n	251
Gender, m/f (%)	171/80 (68.1/31.9)
Age, median, range	57, 25–84
Etiology of liver disease	
Alcohol abuse, n (%)	135 (55.8%)
Hepatitis C, n (%)	74 (29.5%)
Hepatitis B, n (%)	34 (13.5%)
NASH ¹ , n (%)	7 (2.8%)
AIH, n (%)	4 (1.6%)
Hemochromatosis, n (%)	7 (2.6%)
Cryptogenic, n (%)	23 (9.2%)
HCC	
Diagnosed HCC	46 (18.3%)
Child-Pugh stage	
A, n (%)	51 (20.3%)
B, n (%)	118 (47.0%)
C, n (%)	82 (32.7%)
MELD², median, range	15 (6–40)
Treatment	
Liver transplantation, n (%)	30 (12.0%)
Laboratory results	
Sodium (mmol/l), median, range	138, 111–150
ALT ³ (U/l), median, range	32, 2–1594
AST ⁴ (U/l), median, range	53, 15–2823
GGT ⁵ (U/l), median, range	104, 14–1178
ALP ⁶ (U/l), median, range	116, 31–688
Bilirubin (mg/dl), median, range	2.0, 0.2–26.8
Albumin (mg/dl), median, range	3.2, 1.6–5.2
INR ⁷ , mean, median, range	1.41, 0.89–4.2
Creatinine (mg/dl), median, range	1.02, 0.38–6.77
CRP ⁸ (mg/dl), median, range	1.19, 0.03–16.84
Hb (mg/dl), median, range	10.5, 6.0–18.0
HbA1c (%), median, range	5.3, 3.7–9.8
Thrombocytes (x10 ⁹ /L)	98, 17–1507
Leukocytes/nl	5.23, 0.63–56.63

Abbreviations:

- ¹NASH, non-alcoholic steatohepatitis;
- ²MELD, model of end stage liver disease;
- ³ALT, alanine aminotransferase,
- ⁴AST, aspartate aminotransferase;
- ⁵GGT, gamma-glutamyl-transferase;
- ⁶ALP, alkaline phosphatase;
- ⁷INR, internationalized ratio;
- ⁸CRP, C-reactive protein.

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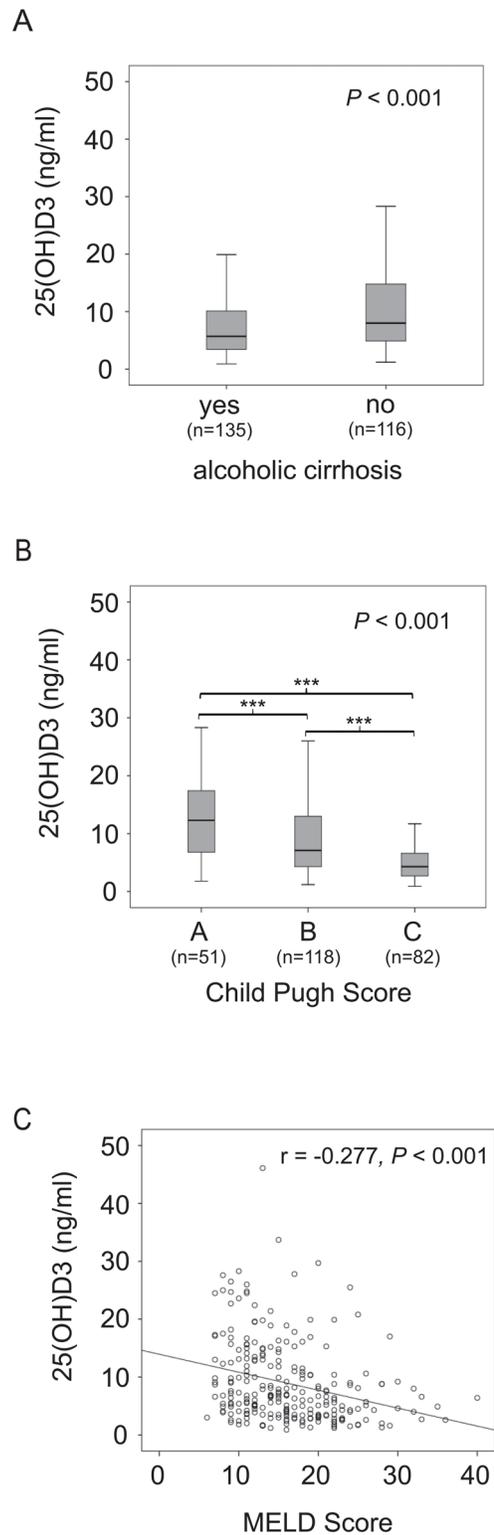


Fig 1. Association of Vitamin D levels with etiologies and stages of cirrhosis. Vitamin D levels in patients with and without alcoholic liver disease (1A). Serum 25(OH)D₃ concentrations in stages of cirrhosis (1B) and its correlation with the MELD score (1C).

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in male compared to female patients ($P = 0.311$). Serum 25(OH)D₃ levels did not significantly differ between blood samples acquired during summer/spring or winter/autumn time ($P = 0.369$).

25(OH)D₃ levels differ among stages of cirrhosis

It has been shown that 25(OH)D₃ levels decrease with the progress of cirrhosis. Therefore, 25(OH)D₃ levels were compared between stages of cirrhosis. As shown in Fig 1B, there were significant differences among Child Pugh scores with the highest levels in Child A and the lowest levels in Child C patients ($P < 0.001$). In addition, the relation between 25(OH)D₃ levels and the MELD score was assessed. There was a significant inverse correlation between 25(OH)D₃ concentrations and the MELD score ($r = -0.277$, $P < 0.001$) (Fig 1C), indicating a relation between severity of cirrhosis and low 25(OH)D₃ levels. 192 patients had decompensated cirrhosis at the day of study inclusion coming along with portal hypertension, ascites, hepatic encephalopathy or varices. Ascites and HRS are typical complications of liver insufficiency and portal hypertension, indeed patients without or with moderate ascites had significantly higher 25(OH)D₃ levels compared to individuals with massive ascites ($P < 0.001$). Individuals with HRS, a severe complication of cirrhosis, had significantly lower 25(OH)D₃ levels in contrast to subjects without HRS ($P < 0.001$) (Data not shown).

25(OH)D₃ and infectious complications

Bacterial infection is a frequent cause of hepatic decompensation. 84 of the 251 patients (33.5%) presented with bacterial infections on the day of study inclusion, namely SBP ($n = 20$), urinary tract infections ($n = 41$), pulmonary infections ($n = 16$) and other infections ($n = 7$). Patients suffering from infections at the day of study inclusion had significantly lower 25(OH)D₃ levels compared to individuals without infections ($P < 0.001$) (Fig 2A). SBP is a severe infectious complication with a high mortality among cirrhotics. Patients with SBP had significantly lower 25(OH)D₃ levels than patients without SBP ($P = 0.011$) (Fig 2B). As vitamin D levels were lower in patients with clinical apparent infections and were reported to be inversely correlated with the activation of the innate immune system in chronic liver diseases (9), the relationship of the surrogate parameters for systemic inflammation, namely CRP and sCD163, were compared to 25(OH)D₃ levels. As shown in Fig 2C and 2D, 25(OH)D₃ levels correlated inversely with CRP ($r = -0.133$, $P = 0.036$) and sCD163 levels ($r = -0.280$, $P < 0.001$).

Low 25(OH)D₃ levels are an independent risk factor for mortality in cirrhotics

As patients with advanced cirrhosis had lower serum 25(OH)D₃ concentrations, we hypothesized that 25(OH)D₃ levels might be of prognostic value in these patients. Recently, serum 25(OH)D₃ concentrations of 6 ng/ml or 10 ng/ml indicating 25(OH)D₃ deficiency have been proposed to be of prognostic value in cirrhotic individuals with or without HCC, respectively [14,15,19]. 25(OH)D₃ levels ≤ 6 ng/ml were associated with higher mortality compared to higher 25(OH)D₃ levels in the present cohort (HR 1.723, 95% CI 1.122–2.646, $P = 0.013$ for 6 ng/ml). However, serum 25(OH)D₃ concentrations ≤ 10 ng/ml were not significantly associated with higher mortality HR 1.424, 95% CI 0.889–2.279, $P = 0.141$ for 10 ng/ml) (Fig 3). Several patients received vitamin D substitution. Therefore, the impact of vitamin D alimentation was investigated. Substitution with vitamin D was not significantly associated with overall survival (HR 0.841, 95% CI 0.473–1.494, $P = 0.555$).

To further analyze if serum 25(OH)D₃ level might be an independent prognostic parameter a multivariate Cox regression model with forward stepwise likelihood ratio was performed.

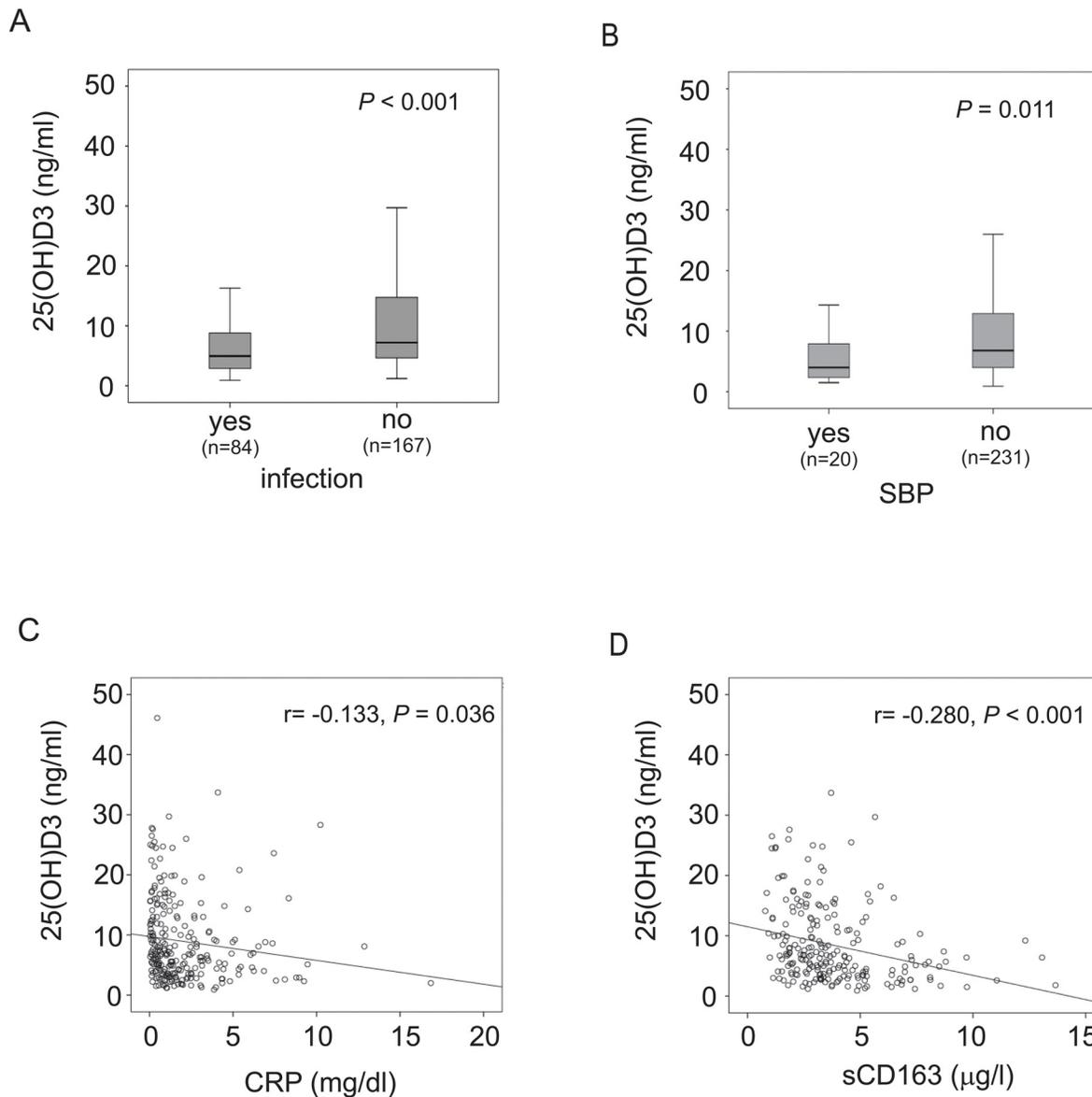


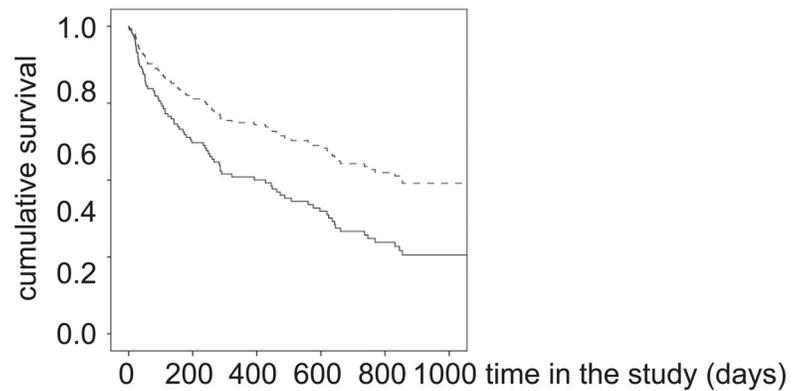
Fig 2. Relation of 25(OH)D₃ levels and infections. Serum Vitamin D levels in patients with and without infections (2A) and spontaneous bacterial peritonitis (2B). Relations of 25(OH)D₃ concentrations and C-reactive protein (CRP) levels (2C) as well as soluble CD163 (sCD163) concentrations (2D) are shown.

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The nominal variables age (≤ 57 years vs. > 57 years), gender, HCC, serum sCD163 levels (≤ 3950 ng/l vs. > 3950 ng/l), presence of infection, Child Pugh score (A,B,C) as well as the continuous variables MELD score and 25(OH)D₃ levels were included in the analysis. As shown in Table 2 25(OH)D₃ levels < 6 ng/ml, HCC, infections and the MELD score were parameters independently associated with mortality.

Discussion

Vitamin D has manifold functions including bone homeostasis and regulation of immune responses. In the present study, we showed that serum 25(OH)D₃ correlated with the severity of liver insufficiency and were associated with infectious complications. Furthermore, very low



25(OH)D₃ > 6 ng/ml 138 89 71 54 41 21 patients at risk
25(OH)D₃ ≤ 6 ng/ml 113 48 37 28 19 4 patients at risk

Fig 3. Relation of Vitamin D concentrations and mortality. The overall survival times of patients with the indicated Vitamin D levels are shown.

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serum 25(OH)D₃ concentrations were an independent risk factor for mortality in patients with cirrhosis in addition to the MELD score, HCC and infections.

Liver fibrosis and especially cirrhosis are linked to low 25(OH)D₃ levels in alcoholic liver disease as well as in HCV related disease with and without HIV coinfection [14,20,21]. In accordance with previous reports patients with alcoholic related cirrhosis showed lower 25(OH)D₃ levels compared to patients with other etiologies of advanced liver disease [14,22]. The reasons for particularly low vitamin levels in individuals with alcoholic cirrhosis are unknown. An alimentary deficit itself might be causative. We found in our cirrhotic patients a negative relation of liver function, namely MELD score and Child Pugh score, and serum vitamin D

Table 2. Univariate and multivariate analyses of parameters associated with mortality.

Parameter	Univariate analysis			Multivariate analysis		
	HR ¹	95% CI ²	P value	HR	95% CI	P value
Male gender	1.407	0.902–2.196	0.133			
Age > 57 years	1.191	0.775–1.832	0.425			
MELD score ³	1.098	1.063–1.135	< 0.001	1.096	1.055–1.140	< 0.001
Log 25(OH)D ³	0.422	0.215–0.828	0.012			
25(OH)D ³ < 6 ng/ml	1.723	1.122–2.646	0.013	1.703	1.038–2.794	0.035
HCC ⁴	2.187	1.328–3.602	0.002	3.399	1.762–6.558	< 0.001
sCD163 ⁵ ≤ 3950ng/l	0.360	0.227–0.572	< 0.001			
Infection	2.706	1.749–4.188	< 0.001	1.878	1.140–3.093	0.013
Child Pugh A vs. C	0.273	0.137–0.543	< 0.001			
Child Pugh B vs. C	0.606	0.381–0.963	0.034			

Abbreviations:

¹HR, hazard ration;

²CI, confidence interval;

³MELD, model of end stage liver disease;

⁴HCC, hepatocellular carcinoma;

⁵sCD163, soluble CD163.

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concentrations, which has also been observed in other cohorts of patients suffering from liver diseases [23]. Severe liver insufficiency is a reasonable cause for disturbance of the 25(OH)D₃ generation. The hydroxylation of cholecalciferol to its bioactive form, namely 25(OH)D₃, is carried out by the liver, showing its key role in vitamin D metabolism [8,9]. This hypothesis is supported by the lack of difference between the 25(OH)D₃ levels determined either in spring/summer or autumn/winter, indicating that an insufficient hydroxylation of cholecalciferol rather than a lack of sun exposure might be the main reason of vitamin D deficiency in cirrhotic patients. An uncoupling of vitamin D levels and sun exposure has recently been found in patients suffering from HCC [19]. Furthermore, patients with decompensated cirrhosis had lower serum 25(OH)D₃ levels compared to patients with compensated disease. Similarly to a previous report, patients with the severe complication of cirrhosis, SBP had significantly lower serum vitamin D [24]. Additionally, stressing the relation of vitamin D deficiency and advanced cirrhosis patients with complications of portal hypertension such as massive ascites and HRS had lower levels of serum 25(OH)D₃ compared to individuals without the indicated complications.

A common cause of hepatic decompensation in cirrhotic patients is the occurrence of bacterial infections [25]. 25(OH)D₃ modulates inflammatory responses and restrains innate immune responses [26,27], e.g. it limits the activation of monocytes and thereby reduces the production of pro-inflammatory cytokines including tumor necrosis factor α (TNF α) [27]. TNF α plays a critical role in the inflammatory environment and it is considered to be one of the main factors leading to cirrhosis [28]. Experimental studies support the idea of a suppressive effect of 25(OH)D₃ on TNF α release [29–31]. A central pathogenic factor in cirrhosis is bacterial translocation from the gastrointestinal tract into the portal system, leading to sustained inflammatory responses and progression of liver disease. The translocated bacteria may also cause direct infectious complications. Vitamin D protects the gut barrier and prevents bacterial translocation [24,32,33]. Moreover, an increased portal hypertension is associated with lower 25(OH)D₃ levels in patients with alcoholic liver disease [14]. In the present cohort of cirrhotic patients, serum vitamin D concentrations were not only inversely correlated with surrogate parameters of inflammation such as CRP or sCD163, but they were also lower in individuals with clinical apparent infections including SBP.

Low vitamin D levels do not only correlate with the extent of liver dysfunction but are also associated with a less favorable outcome [15,23,34,35]. Recent reports showed their association with higher mortality in alcoholic liver disease [14] as well as cirrhosis of other origins [15]. However, the published studies analyzed rather small cohorts of patients [15] or included only individuals with alcoholic liver disease [14]. Our results that 25(OH)D₃ levels inversely correlated with the MELD score are in agreement with recent data [15,23] showing that serum 25(OH)D₃ decreases with increasing degrees of liver insufficiency and therefore with prognosis. Though, we found in the present study that serum 25(OH)D₃ was an independent risk factor for mortality in addition to the stage of cirrhosis, HCC and infectious complications. To our knowledge, our prospectively recruited cohort is the largest cirrhosis cohort published so far investigating the relation of serum vitamin D levels and overall survival in a prospective fashion.

In conclusion vitamin D is not only associated with the severity of liver disease and infections, but also an independent prognostic parameter. Whether 25(OH)D₃ supplementation may improve prognosis of cirrhotic patients warrants prospective interventional trials.

Author Contributions

Conceived and designed the experiments: FF BK AP OW. Performed the experiments: FF. Analyzed the data: FF BK AP OW. Wrote the paper: FF BK SZ AP OW.

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