Original Paper

Correlations of Serum Vitamin D Level with Markers of Oxidative Stress and Apoptosis in Liver Cirrhosis

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ABSTRACT. In this study we investigated the relationship between vitamin D and markers of oxidative stress and apoptosis in patients with liver cirrhosis stratified according serum GGT activity. Forty-eight patients with liver cirrhosis of various aetiology were selected, among which 58% cases (n=28) diagnosed with alcoholic liver cirrhosis and 42% (n=20) with cirrhosis after hepatitis virus infection. Each group was divided into three quartiles according GGT activity. 25-hydroxyvitamin D [25-(HO) vit D], markers of oxidative stress (catalase, superoxide dismutase) and apoptosis (M30) were compared. Higher levels of GGT were correlated with elevated AST, ALT and ALP values in both groups. A statistically significant difference was observed when comparing 25-(OH) vit D levels of patients suffering from ethanol-induced liver cirrhosis versus control group for all the quartiles as well as for those from the first quartile of viral-induced liver cirrhosis. For SOD, statistically significant differences were noticed between all cirrhosis subgroups and the control group. CAT values in all cirrhosis subgroups were lower than in control, but significant differences were only between Q2.2 and Q1.3 quartiles and Q2.2 and control. Correlation of 25-(OH) vit D versus SOD yields statistically significant results in ethanol-induced cirrhosis patients. M30 activity was increased in patients with alcoholic cirrhosis compared to controls and those with virus-induced cirrhosis, being correlated with the degree of GGT activity. Our results emphasized that vitamin D deficiency is associated with enhanced liver dysfunction regardless of the trigger responsible for disease onset. Furthermore, vitamin D deficiency augments liver injury by promoting oxidative stress which influence the survival mechanisms of parenchymal liver cells.

KEYWORDS: *Alcohol-induced liver cirrhosis, HBV/HCV-induced liver cirrhosis, 25-(OH) vitamin D, oxidative stress, apoptosis.*

Introduction

The liver is one of the most important organs due to the various functions it holds.

Many biochemical processes and functions developed in the liver are associated with the generation of various harmful oxygen species as by-products [1].

Oxidative stress reflects the imbalance between the bioavailability of reactive oxygen/nitrogen species (ROS/RNS) and limited antioxidant defences.

At low levels, ROS have physiological roles, but at high concentrations they can interact with cellular components, such as proteins, lipids and DNA.

DNA damage can result in chromosomal instability, altered gene expression, genetic changes linked to cell death, and further liver damage.

The oxidation of proteins and lipids can be responsible for the way some enzymes, structural proteins, and cell membranes operate [2,3].

Numerous studies indicate that oxidative stress brought on by increased ROS/RNS generation has a role in the development of more than 200 diseases, ranging from malignancies to male infertility, Alzheimer's disease, and metabolic disorders, like insulin resistance and diabetes [4-6].

Remarkably, all chronic liver disorders, independently from their aetiology, has in common a highly oxidative milieu that maintains cellular damage, trigger inflammation, activate hepatic stellate cells and contributes to the progression of fibrosis, cirrhosis, and finally hepatocellular carcinoma [7,8].

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Alcoholic liver disease is associated to an exacerbated ROS production and the onset of oxidative stress in hepatocytes due to the metabolic biotransformation of alcohol in the liver [9].

Oxidative stress is also involved as a pathogenic mechanism in viral hepatitis.

HBV and HCV infections are characterized by increased liver and blood levels of various oxidative stress markers that trigger inflammatory pathways activation and cause apoptosis and necrosis of hepatocytes.

As pro-fibrotic mediators generated in hepatocytes, HSCs, and macrophages, ROS stimulate the activation of myofibroblasts responsible for collagen synthesis and excessive accumulation of extracellular matrix (ECM) [10].

Oxidative stress-induced chemical modification of biomolecules leads to their functional inactivation preceding liver cell apoptosis [11].

Glutathione antioxidant system is considered a key player among the antioxidant mechanisms which fight against the oxidative stress.

γ-Glutamyl transpeptidase (GGT), a protease found also on the external surface of the epithelial cells from the biliary ducts, is the only enzyme able to catalyse the degradation of extracellular glutathione [12] and it has long considered as a marker of liver damage.

Several studies outline the elevated serum GGT activity as a robust biomarker of increased body oxidative stress [13,14].

Oxidative conditions induce apoptosis of the lining epithelial cells of the bile ducts [15] and allow the release of GGT.

GGT serves as both an antioxidant and a prooxidant because outside the cells its products are able to reduce ferric to ferrous ions producing ROS as coproducts of this reduction [16].

In a pilot study carried out in order to assess the relationship between serum GGT, GSH, GPx and lipid peroxides we revealed different profiles for the interdependence between GGT activity and the markers of oxidative stress in liver cirrhosis induced by various etiological triggers (ethanol consumption and viral infection).

Also, we report that even in its normal range, GGT might be an early and sensitive marker related to oxidative stress.

Although GGT is recognized as a biomarker of liver injury even in conditions of oxidative stress and many studies have been conducted on the association between liver pathology and vitamin D, few researches refer to the link between GGT, vitamin D status and oxidative stress in these conditions.

Vitamin D is a fat-soluble vitamin synthetized in the skin, from cholesterol under UV exposure, or absorbed from foods or pills in the gastrointestinal tract.

Afterwards it is transported to the liver where is hydroxylated by 25-hydroxylase and 25-hydroxyvitamin D [25-(HO) D], a stable derivative, is formed.

Besides its involvement in the regulation of bone and muscle activity, vitamin D (cholecalciferol) has also extra skeletal functions.

Recent research data has demonstrated pleiotropic effects of vitamin D in different cells and tissues [3,17].

Vitamin D receptors are identified in many organs, such as the pancreas, intestines, muscles, and nervous tissue [18].

In addition to the involvement in calcium and phosphorus homeostasis, vitamin D is important for regulation of hormone secretion, cell proliferation and differentiation.

In the last years, vitamin D anti-inflammatory, antiapoptotic, and antifibrotic roles have aroused the interest of researchers.

Vitamin D deficiency is associated with many diseases (metabolic and autoimmune diseases, heart dysfunctions, chronic infections and cancer) [19].

Vitamin D deficiency is frequent in chronic liver diseases [4].

The earliest reports of disturbed vitamin D metabolism in liver cirrhosis appeared in the late 1970 being attributed to inadequate liver function related to 25-hydroxylation [20].

Since then, many researches were focused on the relationship of vitamin D deficiency and liver fibrosis.

Malham et al. estimated that about one-third of cirrhotic patients have vitamin D deficiency associated rather with organ dysfunction than with disease aetiology being not considered prevalent only to cholestatic disorders [21].

The primary aim of our study was to found vitamin D deficiency in patients with alcoholic liver cirrhosis and cirrhosis developed as a final step of viral hepatitis included in our study groups and to evaluate its relationship with oxidative stress and apoptosis, pathogenic mechanisms responsible for disease progression.

We assess the correlations between serum vitamin D level, superoxide dismutase (SOD) and catalase (CAT) activity, as markers of oxidative stress, and M30, as a marker of epithelial cells

apoptosis, in selected patients with liver cirrhosis stratified according serum GGT levels.

Materials and Methods

Statement of Ethics

The institutional Ethics Committee of the University of Medicine and Pharmacy of Craiova approved the study in accordance with the European Union Guidelines (Declaration of Helsinki) (registration number 116/11.11.2019), and the registered participants provided their written informed consent to be included.

Patients

Forty-eight patients with compensated or decompensated liver cirrhosis hospitalized at the First Clinic of Internal Medicine, Clinical City Hospital "Filantropia" and the Second Clinic of Internal Medicine, Emergency Clinical County Hospital of Craiova, Romania from November 2019 to February 2020 and ten age-matched healthy volunteers were included in this study.

The medical history, clinical examination, laboratory testing, ultrasonography, and endoscopy were used to make the diagnosis.

The following were listed as exclusion criteria: usage of corticoids or non-steroidal anti-inflammatory drugs, pregnancy, drug abuse, comorbid conditions that could worsen systemic inflammation (such as diabetes, metabolic syndrome, inflammatory and autoimmune disorders), and drug misuse.

Our study's eligible individuals were split into two groups: group 1 had cirrhosis developed after alcoholic liver disease (n=28), and group 2 had non-alcoholic liver cirrhosis that resulted from HBV and HCV infection (n=20).

The length of time of disease progression was noted for each subject.

Sample collection and laboratory determinations

Blood samples were harvested in vacuum test tubes in the morning, both with lithium heparin and without any anticoagulant (Becton Dickinson, USA) according to the recommendations for each analysis.

Without using an anticoagulant, blood was drawn and left to clot at room temperature for 30 minutes.

After 10 minutes of centrifugation at 3500rpm (Eppendorf 5417R centrifuge) the serum was recovered and either used to assess common biochemical markers or aliquoted in Eppendorf tubes for further analysis of serum vitamin D and M30 levels.

Using an automated analyser Architect c8000 (Abbott Diagnostics, USA), total serum proteins, albumin, alanine and aspartate aminotransferases (ALT, AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) were assessed.

25-(OH) vitamin D measurement

Serum concentration of the metabolite 25-(OH)-cholecalciferol [25-(HO) vit D] was used to assess the vitamin D level.

The analysis was performed in the clinical laboratory of the Emergency Clinical County Hospital of Craiova, using an automated Roche Cobas E601 platform and Elecsys vitamin D total II reagent (Roche Diagnostics GmbH, Germany) based on the electrochemiluminescence immunoassay.

Measurement of superoxide dismutase (SOD) activity

0.5mL of whole blood collected in vacutainers containing lithium heparin were centrifuged for 10 minutes at 3500rpm, the plasma was aspirated and the cells were then washed four times with physiological saline and centrifuged after each wash.

Up to 2mL of cold, redistilled water was added after the final wash, strongly vortexed, and kept at 4°C for 15 minutes to lyse erythrocytes.

Following dilution with 0.01 M phosphate buffer pH 7, the lysate was used for analysis.

SOD activity was determined by measuring the rate at which superoxide produced by the oxidation of xanthine with xanthine oxidase at 505nm inhibited the conversion of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium to a formazan red dye (Ransod kit, Randox Laboratories Ltd., UK).

Measurement of catalase activity

Blood collected in the vacutainers using lithium heparin was centrifuged at 4,000rpm for 15 minutes at 4°C and the resulting plasma was stored on ice.

If not used during the same day, the plasma was frozen at-80°C.

Sample catalase decomposed a known amount of H_2O_2.

The reaction was stopped with a specific inhibitor. In the presence of horseradish peroxidase, residual H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone to give a quinoneimine dye with a maximum absorption at 520nm, the colour intensity being inversely correlated to the amount of catalase in the original sample.

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Measurement of apoptosis marker M30

A single-step solid-phase sandwich immunoassay (M30 Apoptosense, ELISA PEVIVA) was used to evaluate the serum levels of soluble fragments of cytokeratin K18 that contain the M30 neoepitope (K18asp396) which is involved in apoptosis.

The measurement range of this method is 20 to 1000U/L, with a minimum detectable level of 20U/L for M30.

Statistical analysis

The software GraphPad Prism 5.0 was used to examine the data (GraphPad, San Diego, USA).

Mean values were supplied for variables with a normal distribution.

Furthermore, were determined the standard deviation (SD) and standard error of the mean (SEM).

Median values for non-normal variables were reported (interquartile range-IQR).

The One-Way ANOVA test was used to determine whether there were any significant differences between the study groups for normally distributed data.

A Pearson correlation coefficient of <0.05 was regarded as a significant value.

Results

Forty-eight patients suffering from liver cirrhosis were included in this observational study, of which 28 patients (group 1) developed the disease due to chronic ethanol consumption and 20 patients developed the disease as a consequence of chronic hepatitis B virus (HBV)/hepatitis C virus (HCV) infection (group 2).

The two groups were further subdivided based on the values obtained for serum GGT activity in the following quartiles (quartile 1 (Q1)≤61U/L, quartile 2 (Q2) 61-80IU/L and quartile 3 (Q3)>80IU/L).

These subgroups were then analysed based on the mean±standard error of mean (SEM), median and interquartile range (IQR).

The values of baseline parameters assessed for the two groups of patients divided on the basis of GGT quartiles are presented in Table 1 and Table 2.

Table 1. Baseline characteristics of the patients with ethanol-induced live cirrhosis (Group 1) divided on the basis of GGT quartiles.

Characteristics	Subgroup 1.1 (Q1.1) (GGT level ≤61IU/L)			Subgroup 1.2 (Q1.2) (GGT level 61-80IU/L)			Subgroup 1.3 (Q1.3) (GGT level >80IU/L)		
	Mean ± EM	*Median (Q1, Q3)*	*IQR*	*Mean ± SEM*	*Median (Q1, Q3)*	*IQR*	*Mean ± SEM*	*Median (Q1, Q3)*	*IQR*
AST (IU/L)	34.6 ± 9.37	31 (18.5-52.5)	34.0	43.44 ± 6.87	34 (30.0-61.0)	31	96.77 ± 20.05	78 (49.0-134.0)	85
ALT (IU/L)	25.33 ± 2.89	24.5 (19.5-30.3)	10.8	26.67 ± 3.56	24.0 (19.0-36.5)	17.5	47.62 ± 8.02	38 (28.0-66.5)	38.5
ALP (IU/L)	206 ± 90.7	106 (73.5-388.5)	315	183.4 ± 38.57	135 (98.0-261)	163	230 ± 57.37	170 (91.0-315.5)	224.5
Total Protein (g/dL)	7.67 ± 0.54	7.2 (6.75-8.82)	2.07	6.99 ± 0.31	7.1 (6.45-7.7)	1.25	7.15 ± 0.27	7.3 (6.85-7.4)	0.55
Albumin (g/dL)	3.97 ± 0.28	4.1 (3.43-4.44)	1.01	2.87 ± 0.38	2.4 (2.4-3.2)	0.8	3.02 ± 0.22	2.8 (2.55-3.4)	0.85

Q-quartile, SEM-standard error of mean, IQR-interquartile range, GGT-gamma glutamyl transferase, ALT-alanine aminotransferase, AST-aspartate aminotransferase, ALP-alkaline phosphatase.

Table 2. Baseline characteristics of the patients with virus-induced liver cirrhosis (Group 2) divided on the basis of GGT quartiles.

Features	Subgroup 2.1 (Q2.1) (GGT level ≤61IU/L)			Subgroup 2.2 (Q2.2) (GGT level 61-80IU/L)			Subgroup 2.3 (Q2.3) (GGT level> 80IU/L)		
	Mean ± SEM	*Median (Q1, Q3)*	*IQR*	*Mean ± SEM*	*Median (Q1, Q3)*	*IQR*	*Mean ± SEM*	*Median (Q1, Q3)*	*IQR*
AST (IU/L)	40.8 ± 4.60	37 (33.5-50.0)	16.5	25.98 ± 0.14	26 (25.8-26.2)	0.4	64.00 ± 7.98	53 (53.0-80.5)	27.5
ALT (IU/L)	32.2 ± 3.63	29 (27.0-39.0)	12.0	24.96 ± 0.13	25 (24.8-25.2)	0.4	42.4 ± 2.62	41 (37.5-48.0)	10.5
ALP (IU/L)	207 ± 86.23	70 (69.0-415)	346	339 ± 0.16	339 (338.5-339)	0.5	278 ± 50.44	359 (154-360)	206
Total Protein (g/dL)	6.86 ± 0.22	7.2 (6.35-7.2)	0.85	7.76 ± 0.10	7.7 (7.6-7.95)	0.4	6.03 ± 0.54	6.73 (7.7-7.0)	0.7
Albumin (g/dL)	3.1 ± 0.25	3.1 (2.6-3.7)	1.1	2.72 ± 0.08	2.6 (2.6-2.9)	0.3	3.22 ± 0.08	3.16 (3.06-3.4)	0.3

Q-quartile, SEM-standard error of mean, IQR-interquartile range, GGT-gamma glutamyl transferase, ALT-alanyl aminotransferase, AST-aspartate aminotransferase, ALP-alkaline phosphatase.

As we expected, higher levels of GGT were correlated with elevated AST, ALT and ALP values for both groups with the highest values being observed for the Q1.3 and Q2.3 subgroups.

When comparing the two groups, patients from group 1 (with ethanol-induced liver cirrhosis) had higher values for AST while patients with viral-induced liver cirrhosis (group 2) had higher levels of AST and ALP.

In terms of total protein levels, we observed that patients with ethanol-induced cirrhosis presented the highest values for the Q1.1 quartile while the differences between patients with higher GGT values were almost non-existent.

As for patients with viral-related cirrhosis, the highest value was observed in the Q2.2 quartile, followed by Q2.1 and Q2.3.

Regarding albumin, while patients with alcoholic cirrhosis presented the highest value in the Q1.1 quartile, without significant differences between Q1.2 and Q1.3 quartiles, those who suffered from HBV/HCV induced cirrhosis presented almost identical results between Q2.1 and Q2.3 quartiles, lower values being obtained for Q2.2.

First, we analysed the correlations between 25-(OH) vitamin D levels in the experimental groups 1 and 2 versus the control group.

Data revealed a statistically significant difference when comparing the 25-(OH) vitamin D levels of patients suffering from ethanol-induced liver cirrhosis versus the control group for all the quartiles (Q1.1, Q1.2 and Q1.3) involved in our study as well as in the first quartile (Q2.1) for the virus-related cirrhosis group in comparison to the same, non-cirrhotic, control group.

Additionally, when analysing the difference between the two groups of cirrhosis, we observed statistically significant difference between the Q2.2 quartile and all the quartiles in the first group (Q1.1, Q1.2 and Q1.3) and between the Q2.1 and Q2.2 quartiles of the second group.

The results are presented in Figure 1.

Next, we analysed the levels of oxidative stress markers (SOD and catalase) in the two groups.

When comparing SOD values between the quartiles with the control group, we noticed statistically significant differences between all the patients' subgroups and the control group.

Additionally, when comparing quartiles, we observed statistically significant differences between the Q2.3 quartile and all the other quartiles (Figure 2a).

In terms of levels of catalase activity, even if the values in all cirrhotic subgroups were lower than in control, the only statistically significant differences were between the Q2.2 and Q1.3 quartiles and the Q2.2 quartile and the control group (Figure 2b).

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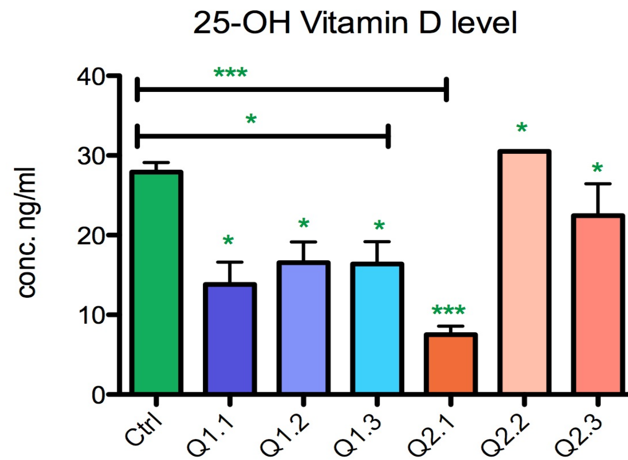

Figure 1. Variation of 25-(OH) vit D level for group 1 (ethanolic liver cirrhosis) and group 2 (viral liver cirrhosis) vs. control group. Mean±SEM Q1.1 vs. Q2.2: 13.81±2.79 vs. 30.51±0.01, p<0.05 (*); Mean±SEM Q1.1 vs. Ctrl: 13.81±2.79 vs. 27.92±1.71 p<0.05 (*); Mean±SEM Q1.2 vs. Q2.2: 16.54±2.62 vs. 30.51±0.01, p<0.05 (*); Mean±SEM Q1.2 vs. Ctrl: 16.54±2.62 vs. 27.92±1.71, p<0.05 (*); Mean±SEM Q1.3 vs. Q2.2: 16.38±2.82 vs. 30.51±0.01 p<0.05 (*); Mean±SEM Q1.3 vs. Ctrl: 16.38±2.82 vs. 27.92±1.71, p<0.05 (*); Mean±SEM Q2.1 vs. Q2.2: 7.51±1.06 vs 30.51±0.01, p<0.05(***); Mean±SEM Q2.1 vs. Q2.3: 7.51±1.06 vs. 22.44±2.99, p<0.05 (*); Mean±SEM Q2.1 vs. Ctrl: 7.51±1.06 vs. 27.92±1.71, p<0.05(***).

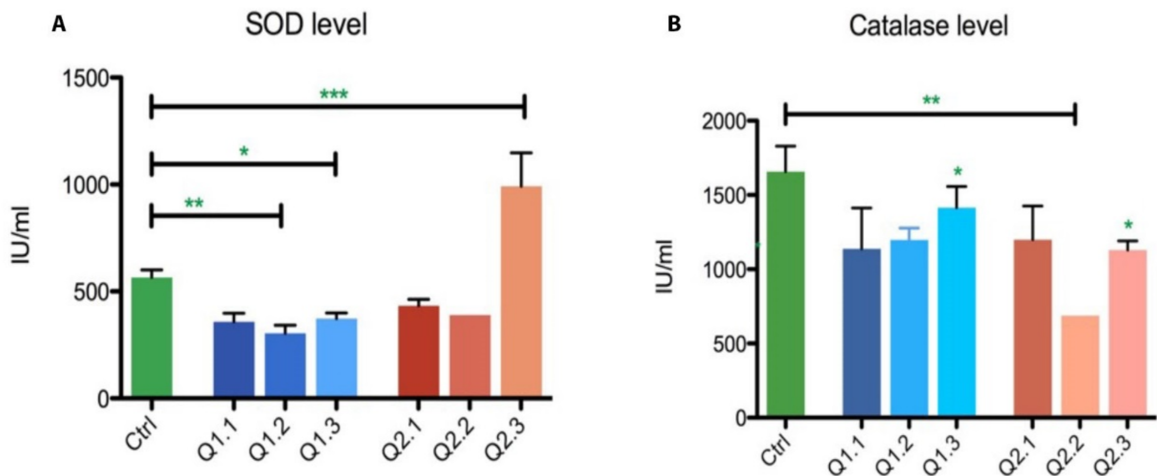

Figure 2. Oxidative stress markers in group 1 (ethanolic liver cirrhosis) and group 2 (viral liver cirrhosis) vs. control group a. SOD activity: Mean±SEM Q1.1 vs. Q2.3: 358.3±39.91 vs. 1130.0±61.76, p<0.05 (***); Mean±SEM Q1.1 vs. Ctrl: 358.3±39.91 vs. 365.8±35.35 p<0.05 (**); Mean±SEM Q1.2 vs. Q2.3: 304.4±67.7 vs. 1130.0±61.76 p<0.05 (***); Mean±SEM Q1.2 vs. Ctrl: 304.4±67.7 vs. 365.8±35.35, p<0.05 (***); Mean±SEM Q1.3 vs. Q2.3: 373.1±25.95 vs. 1130.0±61.76 p<0.05 (***); Mean±SEM Q1.3 vs. Ctrl: 373.1±25.95 vs. 365.8±35.35, p<0.05 (***); Mean±SEM Q2.1 vs. Q2.3: 432.9±30.61 vs. 1130.0±61.76, p<0.05(***); Mean±SEM Q2.2 vs. Q2.3: 389.2±0.08 vs. 1130.0±61.76, p<0.05 (*); Mean±SEM Q2.2 vs. Ctrl: 389.2±0.08 vs. 365.8±35.35, p<0.05(*); Mean±SEM Q2.3 vs. Ctrl: 358.3±39.91 vs. 365.8±35.35, p<0.05(***). b. Catalase activity: Mean±SEM Q1.3 vs. Q2.2: 1415.0±142.4 vs. 687.0±0.1, p<0.05 (*); Mean±SEM Q2.2 vs. Ctrl: 687.0±0.1 vs. 1657±171.2 p<0.05 (**).

We then correlated the levels of 25-(OH) vitamin D levels with the levels of the two oxidative stress markers we previously mentioned.

In terms of group analysis, the comparison yielded statistically significant results for 25-(OH) vitamin D versus SOD in the ethanol-induced cirrhosis patients (p=0.03) and non-significant results for those who were diagnosed with viral-induced cirrhosis (p=0.18) (Figure 3).

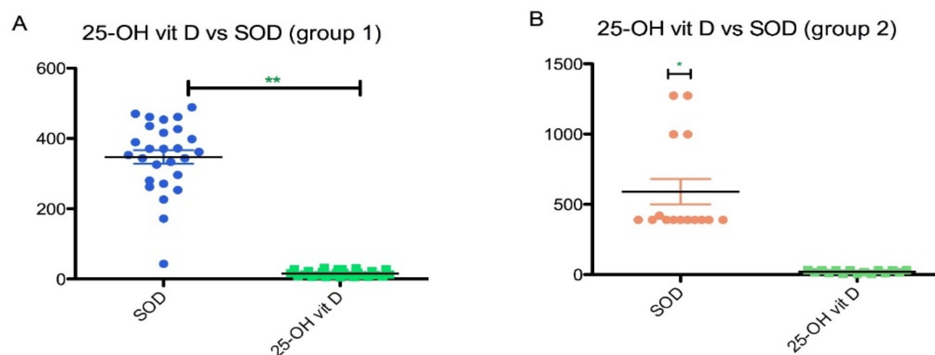

Figure 3. Correlation between 25-(OH) vit D level and SOD activity: A. Group 1, p=0.03 (**); B. Group 2, p>0.05 (ns).

After the group comparison, we performed a subgroup analysis of the correlation between the 25-(OH) vitamin D levels and SOD levels where only two quartiles demonstrated statistically significant results: quartile Q1.3 (p=0.01) and Q2.1 (p=0.02) (Figure 4).

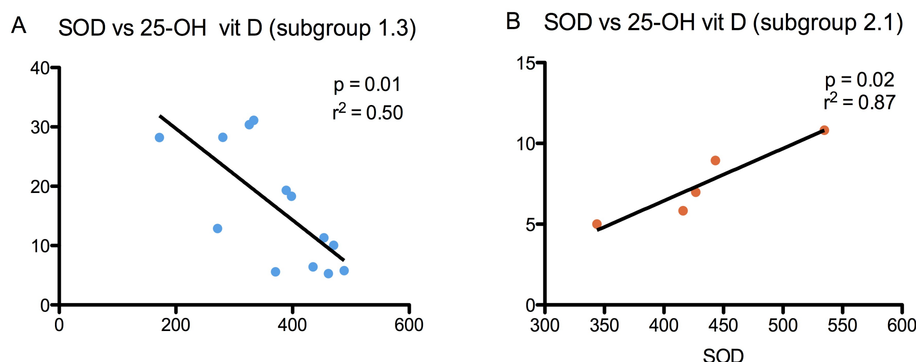

Figure 4. Correlation 25-(OH) vitamin D level vs SOD activity: A. Subgroup Q1.3, p=0.01 (*); B. Subgroup 2.1, p=0.02 (*).

A correlation has been performed between 25-(OH) vitamin D and catalase activity but we didn't reach the statistically significant results due to the limited number of cases (Figure 5).

The subgroup analysis identified only two quartiles where the correlation can be considered significant: Q2.1 and Q2.3 (p=0.01) (Figure 6).

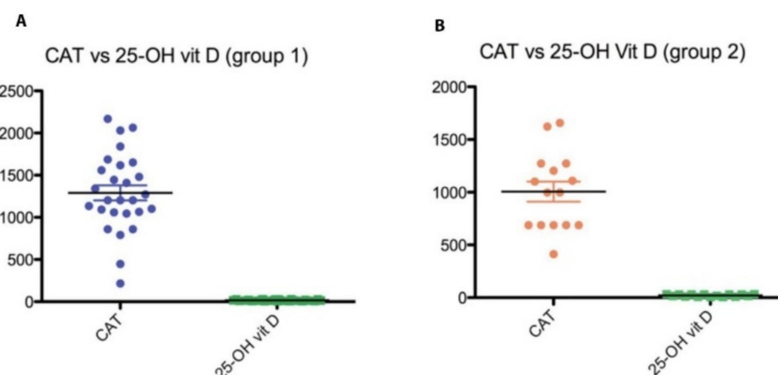

Figure 5. Correlation between 25-(OH) vitamin D and catalase activity: A. Group 1, p>0.05 (ns); B. Group 2, p>0.05 (ns).

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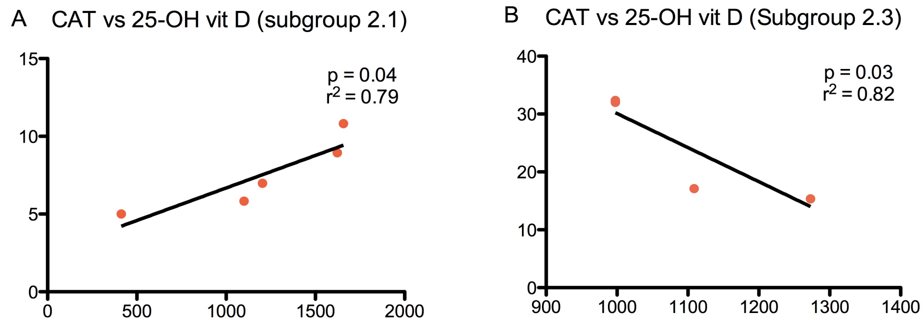

Figure 6. Correlation 25-(OH) vitamin D level versus catalase activity: A. Subgroup Q2.1 p=0.01 (*); B. Subgroup Q2.3 p=0.01 (*).

As for the apoptosis marker M30, the values of activity are increased in patients from group 1 compared to controls and those from group 2, its level being correlated with the degree of GGT activity.

Significant differences were observed when compared M30 values between subgroup Q1.3 and Q2.2 and subgroup Q1.3 and controls ($p<0.05$).

Correlating M30 activity with 25-(OH) vitamin D level and the antioxidant enzymes we noticed significant correlation only between M30 level and catalase activity in subgroup Q2.3.

For this subgroup, a strong correlation was obtained between M30 and the Child-Pugh score, a tool used to assess the prognosis of liver disease, especially cirrhosis (Figure 7).

Also, a strong correlation between M30 and Child-Pugh score was obtained for the group 1 (p=0.002).

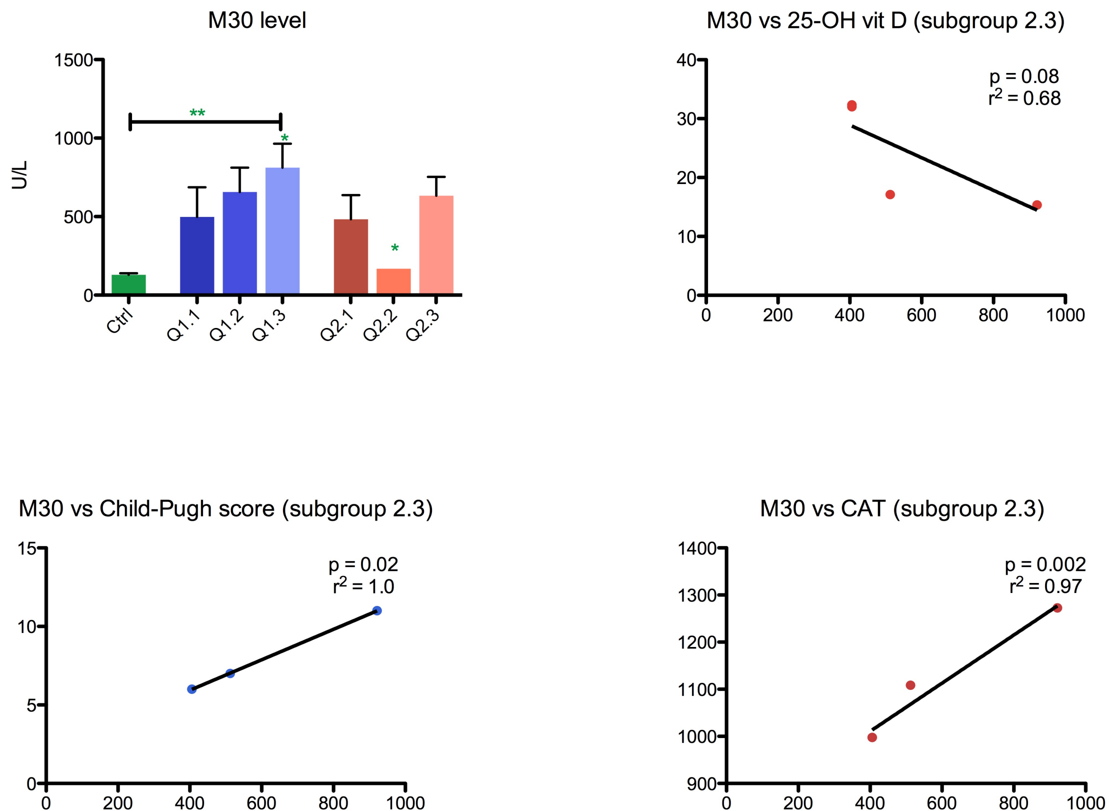

Figure 7. Evaluation of M30 activity: Mean±SEM Q1.3 vs. Q2.2: 811.16±152.6 vs. 167.8±0.17, p<0.05 (*); Mean±SEM Q1.3 vs. Ctrl: 811.16±152.6 vs. 128.±10.8, p<0.05 (**); Correlation M30 vs. CAT in group 2: subgroup Q2.3 p=0.002 (*); Correlation M30 vs. Child-Pugh in subgroup Q2.3 p=0.02 (*).

Discussion

Vitamin D is a crucial nutrient with pleiotropic effects in health and many data evidence its predictive role in the progression of various diseases, including not only bone disorders, but also autoimmune and infectious diseases, malignancies [22].

Liver disease has also been strongly linked to vitamin D deficiency [23].

Despite numerous studies using human liver cell cultures and animal models and the use of vitamin D supplements, patient clinical data are still not conclusive.

In this observational study, patients diagnosed with liver cirrhosis were included and changes in vitamin D level, liver function and markers of oxidative stress and apoptosis were investigated.

First, our study aimed to document vitamin D deficiency in the patients with cirrhosis included.

Mean 25-(OH) vitamin D levels were 15.96+/-8.58ng/mL in ethanol-induced cirrhosis patients and 14.44+/-10.89ng/mL in those with cirrhosis developed secondary to HBV/HCV infection (controls: 27.92+/-3.62ng/mL, according to our data).

Because the level of serum 25-(OH) vitamin D can reflect the lack of vitamin in the body [24], we found that only 17% of the cirrhotic patients had sufficient vitamin D stores, the others being either deficient or insufficient in this vitamin.

These results are in line with the data reported in many other studies [20,21,25,26] sustaining the fact that many patients with chronic liver disease suffer from vitamin D insufficiency.

The mean 25-(OH) vitamin D concentrations in cirrhotic patients were lower than in those from the control group, even if the mean value for the control subjects validates the finding that a deficiency of vitamin D could appear even in the healthy population of our country.

We didn't notice significant difference between vitamin D level in patients with ethanol-induced cirrhosis and those with viral infection, this observation being in accordance with literature data that affirm the widespread presence of vitamin D deficiency regardless the etiology of chronic liver disease.

It has been reported that the incidence of vitamin D deficiency increases as the liver disease progresses, being higher in cirrhotic patients with biochemical and clinical changes that place them in Child-Pugh class C [20].

A clinical study found that vitamin D supplementation reduced the Child-Pugh score in patients with ethanol-induced liver cirrhosis [27].

Various pathogenic mechanisms could be discussed to understand the decrease of serum vitamin D level in patients with chronic liver diseases.

First of all, the alteration of its bioavailability due to malnutrition, low intestinal absorption and tissue distribution in order to be activated.

In addition, impaired liver hydroxylation of vitamin D and its increased catabolism [26].

It must be taken into account that vitamin D deficiency is related to ethanol metabolism.

Shankar et al. suggest that ethanol disrupts vitamin D_3 homeostasis, reducing circulating level of 1,25-$(OH)_2$ vitamin D_3 as a result of renal 1,25-$(OH)_2$ vitamin D_3-24-hydroxylase induction mediated by the oxidative stress induced by renal CYP2E1 metabolism of ethanol through MAPK (mitogen activated protein kinase) activation [28].

The association between vitamin D deficiency and disease progression of HBV/HCV infections to liver cirrhosis was claimed by several studies [29-31].

Vitamin D is a key player in the modulation of the innate and adaptive immune response.

Besides its involvement in the production of various molecules able to defend the body against pathogen invasion, it interferes with progression of viral hepatitis by inhibiting HCV production and also HBV transcription and translation [30].

Related to HBV infection other authors mention different vitamin D impacts [32,33].

Oxidative stress is a critical factor in ethanol and virus-induced liver disorders.

Ethanol is metabolized in the liver by different enzymatic systems to acetaldehyde, a very reactive compound able to form adducts with biomolecules generating tissue injury, which is subsequently catabolized to acetate.

Ethanol-induced liver injury is associated with an enhanced ROS production and the onset of oxidative stress in hepatocytes [9].

Oxidative stress is also blamed as a pathogenic mechanism in HBV and HCV infection.

Hepatitis viruses enter into the cells and develop damaging events, including immune-mediated ER oxidative stress, oxidative damage and deregulation of cell signaling pathways induced by viral proteins [8].

In both HBV and HCV infections a wide array of oxidative stress markers is present and able to activate inflammatory pathways [8,34].

In this regard, we evaluated antioxidant enzymes (SOD, CAT, GPx), GSH, lipid

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peroxides and carbonylated proteins as markers of oxidative stress in our studies [35].

In this study, we presented statistically significant differences of SOD activity between all the patients' subgroups and the control group and lower activity of CAT, even if not significant in all quartiles.

Correlating the level of 25-(OH) vitamin D with the levels of the antioxidant enzymes, we obtained statistically significant results for 25-(OH) vitamin D versus SOD in the ethanol-induced cirrhosis patients.

The lack of statistically significance for the correlation 25-(OH) vitamin D versus SOD and CAT in viral-induced cirrhosis group could be related to the limited number of cases included in each quartile.

Literature data have reported the association between low serum levels of vitamin D and increased oxidative/nitrosative stress [36-38].

Through its interaction with vitamin D receptor (VDR) it mediates protective actions against ROS/RNS injuries strengthening antioxidant defence and has anti-inflammatory and antiproliferative effects in liver fibrosis [39,40].

Vitamin D induces the expression of several antioxidants, such as reduced glutathione, GPx and SOD [41].

According to de Almeida et al. [42] insufficient vitamin D level aggravates liver inflammation and oxidative stress in patients with HCV.

Hu et al. demonstrated that vitamin D insufficiency elevated alcohol-induced upregulation of proinflammatory cytokines, attenuated upregulation of hepatic antioxidant enzymes genes, such as *sod* and *gshpx,* and aggravated oxidative stress and inflammation during chronic alcoholic liver disease [43].

It is also widely acknowledged that oxidative stress can cause lipid peroxidation, impaired mitochondrial and peroxisomal fatty acid oxidation, and cytokine release.

Corroborating our studies with literature data, we suggest that cirrhotic patients deficient in vitamin D are more prone to lipid peroxidation and the other oxidative changes due to an impaired action of enzymatic and non-enzymatic antioxidant systems.

These findings imply that oxidative stress caused by reactive species plays a significant role in the aetiology of cirrhosis, which may then be linked to liver cell death.

The release of inflammatory cytokines under extensive ROS/RNS formation causes apoptosis and necrosis of hepatocytes.

In this study we assessed apoptosis in patients from the study groups performing serum caspase-cleaved cytokeratin (M30) measurement.

Cirrhotic patients from ethanol-induced liver cirrhosis group had increased activity of M30 compared to controls and those from virus-induced disease, its level being correlated with GGT activity.

Our results are in accordance with those reported by Oweira et al. who found significantly higher serum levels of M30 and also M65 in cirrhotic patients compared to controls and a good correlation between these markers and disease outcome assessed with MELD score [44].

In our study, a strong correlation was observed between M30 and Child-Pugh score in patients from the ethanol-induced cirrhosis group and for those with increased GGT activity in the virus-induced liver cirrhosis group.

Other team also reported a negative correlation between Child-Pugh score and serum vitamin D concentration [45] but in our study we didn't find significance even if many patients classified as Child-Pugh C presented lower levels of 25-(OH) vitamin D.

The death of hepatocytes occurs in normal conditions, as an adaptive reaction to accomplish the removal of damaged cells, and almost in all types of liver disorders, as a crucial factor in disease progression [8].

As a result of extensive ROS/RNS formation inflammatory cytokines are released and apoptosis and necrosis of liver cells could happen.

Necrotic hepatocytes send danger signals to neighbouring cells and induce the activation of matrix metalloproteinases, leading to fibrosis as an impaired remodelling of ECM.

ROS are powerful inducers of apoptosis triggering both the intrinsic mitochondrial and the extrinsic death receptor pathways in addition to ER stress pathway [46,47].

Caspase activation is just one of the mechanisms through which ROS regulate apoptosis [8].

Literature data sustain the involvement of hepatocytes' apoptosis in viral hepatitis, ethanol-induced and cholestatic liver injury [47,48].

Metabolic and inflammatory factors lead to mitochondrial dysfunction, ROS/RNS production, Bax translocation to mitochondria, and caspase activation [49].

Serum caspase-cleaved cytokeratin fragment M30 (marker of apoptosis) and M65 (marker of necrosis) are clinically useful markers of liver fibrosis and inflammation in patients with chronic liver diseases [44,50].

The studies regarding the predictive and prognostic value of cytokeratin 18 fragment in patients with liver injury reported that M30 and M65 levels are increased in various liver diseases [51-53] being a strong reason for our results.

Vitamin D might mediate positive effects through its anti-apoptotic activity and its long-term insufficiency could activate inflammatory pathways and subsequently higher oxidative stress responsible for enhanced hepatocyte apoptosis [54].

Conclusions

The results emphasized that vitamin D deficiency is associated with enhanced liver dysfunction regardless of the trigger responsible for disease onset.

Furthermore, vitamin D deficiency enhances liver injury by promoting oxidative stress and inflammation which influence the survival mechanisms of parenchymal liver cells.

In conclusion, patients with liver cirrhosis and decreased serum 25-(OH) vitamin D level are more prone to enhanced stress responses, decreased levels of antioxidant enzymes and alteration of apoptosis.

While our data sustain that vitamin D deficiency is commonly found in patients with liver cirrhosis included, being a pilot observational study, further research are necessary to clarify whether vitamin D deficiency affects the progression of liver disease to cirrhosis or liver disease influences vitamin D status.

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Conflict of interests

None to declare.

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