

# Correlation between vitamin D levels and apoptosis in geriatric patients infected with hepatitis C virus genotype 4

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**Background:** Vitamin D levels play a pivotal role in most biological processes and differ according to age. A deficiency of vitamin D in chronic hepatitis C (CHC) patients has been shown to be linked with the severity of liver fibrosis, but little is known about the mechanism of this association.

**Objective:** In this study, we evaluate the potential interrelation between vitamin D levels, oxidative stress, and apoptosis, based on liver fibrosis in geriatric patients infected with hepatitis C virus (HCV) genotype 4.

**Subjects and methods:** A total of 120 adult individuals aged 30–68 years were recruited in this study. Of these, 20 healthy subjects (15 men and five women) with a mean age of 48.3±6.1 years were selected as controls, and 100 patients with a mean age of 47.8±4.9 years with chronic HCV (CHC) who had undergone liver biopsy (80 men and 20 women) were included in this study. Based on liver radiographic (computed tomography, magnetic resonance imaging) and histological Metavir system analyses, the CHC patients were classified into three groups: asymptomatic CHC carriers (n=30), fibrosis (n=25), and cirrhosis (n=45). HCV RNA, HCV genotypes, inflammatory cytokines AFP and TNF $\alpha$ , 25-hydroxyvitamin D (25[OH]D) levels, apoptotic markers single-stranded DNA (ssDNA) and soluble Fas (sFas), and oxidative stress markers nitric oxide (NO) and total antioxidant capacity (TAC) were estimated by using molecular, immunoassay, and colorimetric techniques.

**Results:** Approximately 30% of the study population (n=30) were diagnosed as asymptomatic CHC carriers, and 70% of the study population (n=70) had severe fibrosis; these were classified into fibrosis and cirrhosis. There was a significant reduction in 25(OH)D levels and TAC activity, along with an increase in levels of NO, AFP, TNF $\alpha$ , ssDNA, and sFas in fibrosis and cirrhosis subjects compared with those of asymptomatic CHC carriers and health controls. The deficiency in 25(OH)D levels correlated positively with sFas, ssDNA, AFP, TNF $\alpha$ , NO, and TAC, and negatively with age, sex, liver function, body mass index, homeostatic model assessment – insulin resistance, HCV RNA, and viral load. Significant intercorrelation was reported between serum 25(OH)D concentrations and apoptotic and oxidative markers, which suggested progression of liver pathogenesis and fibrogenesis via oxidative and apoptotic mechanisms.

**Conclusion:** The data showed that vitamin D status was significantly correlated with pathogenesis and fibrogenesis of the liver in geriatric patients infected with HCV genotype 4. The deficiency in 25(OH)D levels was shown to have a pivotal role in the pathogenesis of liver via apoptotic, oxidative stress, and inflammatory mechanistic pathways. The data point to adequate vitamin D levels being recommended for a good response to treatment strategies, especially in older CHC patients.

**Keywords:** 25(OH)D, HCV, apoptosis, Fas antigen, liver fibrosis, oxidative stress, geriatrics



## Introduction

Hepatitis C virus (HCV) is considered one of the major causes of liver injury worldwide.<sup>1</sup> Globally, >170 million individuals suffer from different HCV health problems, ranging from liver injury and cirrhosis to hepatocellular carcinoma (HCC).<sup>2–4</sup> Acute and chronic stages are the most reported stages of HCV infection, with significant appearance of HCV RNA, elevation of serum ALT levels, jaundice, and consequently appearance of HCV antibodies (anti-HCV). In the chronic hepatitis stage, progressive expression of hepatic fibrosis has been reported, ultimately leading to the progression of cirrhosis and HCC.<sup>5</sup>

In HCV-infected patients, oxidant–antioxidant imbalance status has been reported, with a significant increase in reactive oxygen species (ROS).<sup>6</sup> These oxidative free radicals result in more free radical initiation via liver cell-damage mechanisms.<sup>7,8</sup> Most studies have reported that the HCV core protein and nonstructural proteins are responsible for the biological changes in human liver cells, such as an increase in ROS production, inhibition of the electron-transport chain, and altering apoptosis, the transcription process, and cell signaling.<sup>9</sup> Also, these viral proteins provide substantial alterations in endogenous antioxidants and antioxidant enzymes within the human body:<sup>10</sup> an excessive amount of ROS was produced from mitochondria, inflammatory cells, and peroxisomes of the infected liver cells. Besides their role in the promotion of HCV RNA replication and suppression of gene regulation of liver cells, these oxidative free radicals produce more free radicals from the fat and protein constituents of the cell wall and the cell's genetic materials via lipid peroxidation.<sup>11,12</sup> These biologically active oxidative radicals promote the progression of liver-cell fibrosis,<sup>13</sup> and the excessive generation of ROS by HCV infection leads to activation of oncogenic transcription factors and mutagenesis that ultimately ends in a state of HCC.<sup>14</sup>

It has been reported that the integrity of liver tissues depends on homeostatic balance between liver-cell proliferation and apoptosis, and that any disruption of this balance by core proteins of HCV may lead to hepatic carcinogenesis, whereas viral proteins, along with activating transcription of viral and cellular genes, coordinate the balance between proliferation and death processes of liver cells by inducing or blocking apoptosis.<sup>15</sup> In patients with acute and chronic hepatitis (CH), the expression of apoptosis-related proteins was shown to be linked with intrahepatic bile-duct development.<sup>16</sup> The change in 25-hydroxyvitamin D (25[OH]D) levels in the serum of patients with CH is significantly associated with the expression of apoptosis-related proteins, especially in HCC subjects.<sup>17</sup>

Besides its role in the regulation of bones and calcium homeostasis, 25(OH)D is involved in many biological processes.<sup>18</sup> 25(OH)D deficiencies have been reported in hepatitis B/C patients.<sup>19,20</sup> Recent studies suggest a relation of vitamin D status with fibrosis progression and response to the IFN-based therapy.<sup>21</sup> It was reported that during the treatment of CHC with polyethylene glycol–IFN $\alpha$ , considerable amounts of vitamin D administration were shown to increase sustained viral response.<sup>22–24</sup>

Most studies have reported that the response to therapy depends mainly on HCV genotypes,<sup>25</sup> targeting of apoptosis,<sup>26</sup> and 25(OH)D levels in CH patients.<sup>17,27,28</sup> Considerable benefits of therapeutic treatments have been achieved in patients with HCV genotype 2 and 3 infections than genotype 1 or 4, which had significantly lower response rates.<sup>29</sup> Also, vitamin D deficits were significantly reported in HCV genotype 4-infected subjects compared with healthy controls.<sup>30,31</sup>

It has been shown that the hydroxylated form of 25(OH)D controls a variety of genes that regulate more biological processes, including apoptosis and angiogenesis.<sup>32</sup> There was a significant correlation among HCV genotypes, hepatic fibrosis scores, and 25(OH)D deficiency in treated and untreated CHC patients.<sup>33,34</sup>

With regard to age, elderly patients with CHC showed significant decreases in serum vitamin D concentration compared with young patients, especially in elderly women,<sup>21</sup> and young patients were shown to be at lower risk of developing HCC than elderly patients.<sup>35,36</sup> However, little is known about the link between severity of fibrosis, higher 25(OH)D deficiency, and apoptosis as a target for therapeutic treatments in genotype 4 CHC patients. Therefore, in this study, we evaluate the potential interrelation between vitamin D levels, oxidative stress, and apoptosis based upon liver fibrosis in geriatric patients infected with HCV genotype 4.

## Subjects and methods

### Patients

A total of 120 adult individuals aged 30–68 admitted to the outpatient department of the Gastroenterology Surgical Centre, Faculty of Medicine, Mansoura University, Mansoura, Egypt, were recruited in this retrospective study. Of these, 20 healthy subjects (15 men and five women) with a mean age of 48.3 $\pm$ 6.1 years were selected as controls from a population undergoing routine medical investigations for medical insurance, and 100 patients with CHC who had undergone liver biopsy (80 men and 20 women) with a mean age of 47.8 $\pm$ 4.9 years were included in the study. Full reported informed consent was obtained from all participants prior to liver biopsy.

## Patient selection

Patients with proven HCV viremia, HCV RNA positivity, and genotype determinations were selected. Liver biopsy was taken from patients prior to antiviral therapy or any other antifibrotic therapy. Serum marker levels (such as AST, ALT, and AFP) were performed on the day of biopsy or within 5 days after liver biopsy.

Exclusion criteria were presence of HIV and/or HBV coinfection, other causes of chronic liver diseases, HCC, and prior liver transplantation. Also, subjects taking iron supplementation, the overweight and obese (body mass index [BMI]  $\geq 25$  and  $\geq 30$  kg/m<sup>2</sup>, respectively), with previous IFN therapy, and insufficient liver biopsy were excluded from this study.

The study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki and was reviewed and approved by the ethical committee of the Gastroenterology Surgical Centre, Faculty of Medicine, Mansoura University. Also, the study was conducted after obtaining clearance from the institutional ethics committee and written informed consent from each participant. All subjects completed a structured questionnaire with questions regarding demographics and daily medication use. Venous blood samples from each patient were collected either before the administration of preoperative drugs on the day of biopsy or within 5 days after biopsy. Samples were given a coded study identification number and were shipped frozen at  $-80^{\circ}\text{C}$  for analysis.

## Virology

Patients with CHC were diagnosed by elevated levels of ALT and higher titers of anti-HCV, established by third-generation enzyme immunoassay (AxSym HCV 3.0; Abbott Laboratories, Abbott Park, IL, USA). Also, HCV RNA as a measure of diagnosis was reported qualitatively using a nested polymerase chain-reaction Qiagen RNA-extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) and quantitatively using Smart Cycler II real-time polymerase chain reaction (Cepheid, Sunnyvale, CA, USA) with HCV RNA-quantification kits (Sacace Biotechnologies, Como, Italy) for estimation of HCV RNA-positive subjects, as previously described.<sup>37,38</sup> In addition, reverse hybridization was performed to identify HCV genotypes using a line-probe assay (Inno-LiPA HCV II kit; Innogenetics, Ghent, Belgium).<sup>39</sup>

## Laboratory determinations

A previously validated questionnaire<sup>40</sup> was used to collect demographic and medical information. Laboratory test results used in this study were serum ALT, AST, and

blood platelet counts and fasting blood glucose, using standard methods. Serum AFP and 25(OH)D levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) for and immunoassay kits (IDS, Boldon, UK), respectively. Also, TNF $\alpha$  (Origenium Laboratories, Helsinki, Finland) and insulin (Monobind Inc, Lake Forest, CA, USA) were estimated for all participants using ELISA kits. Insulin resistance was determined by homeostatic model assessment (HOMA)<sup>41</sup> using the formula: fasting insulin ( $\mu\text{IU/mL}$ )  $\times$  fasting blood glucose (mg/dL)/405. Serum total antioxidant capacity (TAC) was measured using a colorimetric assay kit (K274-100; BioVision, Milpitas, CA, USA). The data were measured and calculated as previously reported.<sup>42</sup> Plasma NO concentration as measure of oxidative stress parameter was estimated as nitrate and nitrite using a Griess reagent. The concentration of nitrite was measured at 540 nm using high-performance liquid chromatography technology as previously reported.<sup>43</sup> Plasma soluble Fas (sFas; ng/mL) and single-stranded DNA (ssDNA) concentrations were determined as early markers for liver-cell apoptosis using a commercial quantitative enzyme immunoassay kit (Quantikine<sup>®</sup>; R&D Systems) for Fas antigens and an ssDNA ELISA kit (EMD Millipore, Billerica, MA, USA) for ssDNA.

## Histologic examination

After informed consent documents had been submitted, hepatic biopsies were obtained from all cases by a surgeon after computed tomography or magnetic resonance imaging scans. A preoperative clinical diagnosis of primary liver cancer was made on the basis of an elevated serum AFP level ( $\geq 400$  ng/mL) and characteristic features of the disease that were visible in the scans. Histological diagnosis of cirrhosis and HCC was based on international criteria. For histological examination, liver biopsies were obtained using an automatic 16-gauge Trucut needle (biopsy gun), which provides adequate specimens for evaluation and fewer cases with tissue fragmentations. Liver biopsy specimens analyzed were at least 15–25 mm long with complete portal tracts ( $>10$ ). Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin and Masson's trichrome. Slides were labeled with patient-identification numbers and then reviewed and graded blindly by a senior pathologist. The mean length of liver biopsies and the number of portal tracts were assessed (including only the complete, intact portal tracts). The degree of fibrosis was scored according to the Metavir system,<sup>44</sup> and no fibrosis was defined as F0, mild fibrosis as F1, moderate fibrosis as F2, severe fibrosis as F3,

and cirrhosis as F4. Significant fibrosis was also defined as F2–F4. Hepatic inflammatory activity was also scored.

## Statistical analysis

SPSS version 16 was used for statistical analysis. Data are expressed as mean  $\pm$  standard deviation or number (percentage) of patients with a condition. Correlation analysis between the studied variables was done by Student's *t*-test, one-way analysis of variance, and Mann–Whitney *U*-test for continuous variables.

## Results

A total of 120 subjects aged 18–68 years with a mean age of  $48.3 \pm 6.1$  years were recruited in this study. Based on clinical investigations, Anti-HCV titers, HCV RNA, and pathological examination, the subjects were classified into control healthy subjects ( $n=20$ ) and CHC patients ( $n=100$ ), as shown in Table 1. Approximately 70% of CHC patients

showed severe pathological symptoms: they were classified according to fibrosis scores into 25% with fibrosis ( $n=25$ ) and 45% with cirrhosis ( $n=45$ ), and only 30% ( $n=30$ ) of patients were diagnosed as asymptomatic CHC (ASC) carriers. There were significant changes in BMI, increases in serum levels of AST, ALT, and bilirubin, and decreases in albumin in CH patients compared with the control group.

Also, significant changes in serum inflammatory markers, such as AFP and TNF $\alpha$ , along with glucose and HOMA – insulin resistance (IR) were reported in CHC patients compared to control subjects (Table 1). Also, in HCV patients with cirrhosis, there were significant increases in serum levels of AST, ALT, bilirubin, AFP, TNF $\alpha$ , glucose, and HOMA-IR, along with decreases in albumin compared with ASC patients (Table 2). However, significant reductions in levels of AST, ALT, albumin, and HOMA-IR were reported in cirrhotic patients compared with those with fibrosis (Table 2).

With regard to the association between 25(OH)D levels and severity of liver fibrosis, this was significantly lower in CHC patients compared to the control group ( $P \leq 0.05$ ). Also, in cirrhotic patients, the reduction in 25(OH)D levels was shown to be significantly correlated ( $P=0.001$ ) with ASC and fibrosis cases. HCV patients with fibrosis showed lower 25(OH)D levels when compared with either controls or ASC cases. There was significant correlation between lower levels of 25(OH)D and severity of liver fibrosis. HCV-related disease progression was associated with lower median values of 25(OH)D levels compared to nondiseased control patients, while those with cirrhosis had the lowest 25(OH)D concentrations among those with liver disease (Table 3). Also, there was significant association between severity of HCV infection and the deficiency in 25(OH)D levels. In CHC patients with fibrosis and cirrhosis, there were significant increases in viral load, HCV RNA-expression rates, and depletion in 25(OH)D levels compared with the ASC group (Figure 1).

To study the role of inflammation in the pathogenesis of CHC, the cytokines/inflammatory markers AFP and TNF $\alpha$  were estimated in CHC patients. There were significant increases in AFP and TNF $\alpha$  levels among CHC patients with cirrhosis compared with ASC and control subjects, as shown in Table 2.

To study the relationship between oxidative stress status and severity of liver fibrosis, NO and TAC as biomarkers of oxidative free radicals were measured in all participants. All CH patients showed significant increases in NO levels and depletion in TAC activity when compared with

**Table 1** Demographics, parameters studied, and clinical characteristics of geriatric CHC and control subjects

Groups/parameters	Control subjects (n=20)	All CHC patients (n=100)
Age (years) <sup>#</sup>	48.3 $\pm$ 6.1	47.8 $\pm$ 4.9
Sex (male/female) <sup>#</sup>	15/5	80/20
BMI (kg/m <sup>2</sup> ) <sup>#</sup>	23.7 $\pm$ 4.1	24.9 $\pm$ 5.7*
AST (IU/mL) <sup>###</sup>	22.3 $\pm$ 6.3	68.4 $\pm$ 11.2**
ALT (IU/mL) <sup>###</sup>	28.3 $\pm$ 5.6	85.7 $\pm$ 22.3**
Albumin (gm/dL)	4.2 $\pm$ 0.27	2.9 $\pm$ 0.94**
Bilirubin (mg/dL)	0.7 $\pm$ 0.46	3.8 $\pm$ 0.94**
Platelets (109/L) <sup>#</sup>	235 $\pm$ 46.8	187 $\pm$ 24.7**
Duration of HCV (years)	–	7.6 $\pm$ 2.3
HCV RNA (IU/mL)	–	8.3 $\times$ 10 <sup>5</sup>
HCV genotypes	–	G4
Viral load	–	38.7 $\pm$ 6.2
AFP (ng/mL) <sup>###</sup>	3.6 $\pm$ 4.4	25.4 $\pm$ 2.7**
TNF $\alpha$ (pg/mL)	76.6 $\pm$ 35.7	310.0 $\pm$ 52.1**
Glucose (mg/dL)	85 $\pm$ 4.3	98 $\pm$ 6.1*
HOMA-IR	1.85 $\pm$ 0.87	3.15 $\pm$ 1.2*
IR, n (%)	0 (0%)	48 (48%)
ASC/fibrosis/cirrhosis	–	30/25/45
Fatty liver	–	20 (20%)
Portal hypertension and/or ascites	–	35 (35%)
Length of liver-biopsy core	–	20.3 $\pm$ 0.65 cm
Portal tracts	–	15 $\pm$ 5.7
<b>Necroinflammation</b>		
A0–A1	–	30 (30%)
A2–A3	–	70 (70%)

**Notes:** All values represent mean  $\pm$  standard deviation or n (%). “–” indicates these parameters are related to viral estimation and hepatic pathology; <sup>#</sup>Student's *t*-test; <sup>###</sup>Mann–Whitney *U*-test; \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Abbreviations:** CHC, chronic hepatitis C; BMI, body mass index; HCV, hepatitis C virus; HOMA, homeostatic model assessment; IR, insulin resistance; ASC, asymptomatic CHC; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; AFP, alpha fetoprotein; TNF $\alpha$ , tumor necrosis factor- $\alpha$ .



**Table 2** Biochemical profiles of geriatric CHC patients according to liver-fibrosis scores

Groups/ parameters	Control (n=20)	All CHC patients (n=100)			Significance		
		ASC (n=30)	Fibrosis (n=25)	Cirrhosis (n=45)	P <sub>1</sub>	P <sub>2</sub>	P <sub>3</sub>
AST (IU/mL)	22.3±6.3	38.5±5.2 <sup>a</sup>	75.5±12.1 <sup>a</sup>	62.5±6.3 <sup>a</sup>	0.01	0.001	0.001
ALT (IU/mL)	28.3±5.6	35.9±8.3 <sup>a</sup>	98.4±15.3 <sup>a</sup>	48.6±9.4 <sup>a</sup>	0.01	0.01	0.001
Albumin (gm/dL)	4.2±0.27	4.0±0.54 <sup>a</sup>	3.1±0.31 <sup>a</sup>	2.7±0.89 <sup>a</sup>	0.01	0.001	0.001
Bilirubin (mg/dL)	0.7±0.46	0.96±0.84 <sup>a</sup>	2.7±1.4 <sup>a</sup>	4.9±3.4 <sup>a</sup>	0.05	0.01	0.001
Glucose (mg/dL)	85±4.3	92±3.8	85.9±2.7	89.1±3.2	0.05	0.01	0.05
HOMA-IR	1.85±0.87	2.85±1.2	4.38±1.4	4.2±1.1	NS	NS	0.05
IR, n (%)	0	11 (36.7%)	14 (56%)	29 (64.4%)	NS	NS	0.05
AFP (ng/mL)	3.6±4.4	5.4±3.7 <sup>a</sup>	8.5±6.1 <sup>a</sup>	16.7±5.3 <sup>a</sup>	0.01	0.003	0.001
TNFα (pg/mL)	76.6±35.7	20.0±72.1 <sup>a</sup>	24.0±24.7 <sup>a</sup>	29.8±51.3 <sup>a</sup>	0.005	0.001	0.001

**Notes:** All values represent mean ± standard deviation or n (%); <sup>a</sup>significant difference from healthy controls at  $P \leq 0.05$ ; P<sub>1</sub>, comparison of ASC and fibrosis groups; P<sub>2</sub>, comparison of fibrosis and cirrhosis groups; P<sub>3</sub>, comparison of ASC and cirrhosis groups.

**Abbreviations:** CHC, chronic hepatitis C; ASC, asymptomatic CHC; HOMA, homeostatic model assessment; IR, insulin resistance; NS, not significant; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; AFP, alpha fetoprotein; TNFα, tumor necrosis factor-α.

control subjects. However, patients with cirrhosis showed significantly higher increases in NO free radical levels and decreases in TAC activity when compared with patients of ASC and fibrosis groups, as shown in Table 3.

In order to estimate the possible association between apoptosis and liver fibrosis, sFas and ssDNA were estimated as apoptosis-related biomarkers in the plasma of all participants. HCV patients with fibrosis and cirrhosis showed significant increases in plasma levels of sFas and ssDNA when compared with ASC patients and healthy controls, as shown in Table 3; however, significant increases in sFas and ssDNA as apoptosis markers were reported in cirrhotic patients compared with ASC and fibrosis ( $P=0.001$ ) patients. Expression of the apoptosis-related markers ssDNA and sFas showed positive significant correlations with the increase in HCV RNA, viral load, AFP, TNFα, and NO, and decreases in levels of TAC activity (Table 4).

In all CHC patients, deficient 25(OH)D levels showed significant correlations with the parameters studied in all groups. Lower 25(OH)D levels correlated positively with increases in sFas, ssDNA, AFP, TNFα, NO, and decreases

in TAC activity, and negatively with age, sex, liver function, BMI, HOMA-IR, HCV RNA, and viral load in all patients with varying liver fibrosis, as shown in Table 5. Such a relation appeared to have higher significance in cirrhosis patients ( $P=0.001$ ) compared to ASC or fibrosis patients ( $P=0.01$ ).

## Discussion

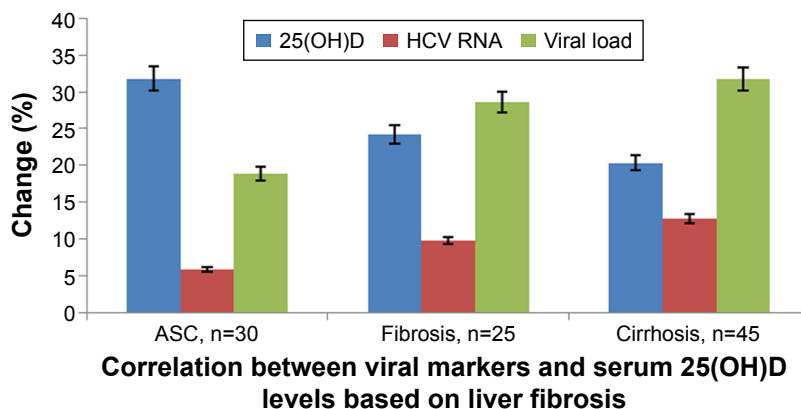
CH is one of the most pathological disorders caused by HCV worldwide. During progression of the disease, there is a significant increase in HCV RNA expression, elevated ratios of viral loads, and significant fibrotic protein deposits.<sup>1,4,5</sup> Vitamin D deficits have been shown to be commonly linked with chronic liver diseases. While 93% of these patients reported insufficient vitamin D levels,<sup>45</sup> almost a third of these showed severe deficiency.<sup>46</sup> The concentration of 25(OH)D in plasma is the most reliable indicator of vitamin D status. It reflects the total amount of vitamin provided from all natural sources, including conversion from fatty deposits of the liver.<sup>47</sup> Therefore, we evaluate the association between 25(OH)D levels, apoptosis, and liver fibrosis in genotype 4 CHC patients.

**Table 3** Change in 25(OH)D levels, oxidative stress, and apoptosis-related biomarkers of geriatric CHC patients based on liver-fibrosis scores

Groups/parameters	Control (n=20)	All CHC patients (n=100)			Significance		
		ASC (n=30)	Fibrosis (n=25)	Cirrhosis (n=45)	P <sub>1</sub>	P <sub>2</sub>	P <sub>3</sub>
Serum 25(OH)D (nmol/L)	38.7±5.8	31.8±3.9 <sup>a</sup>	24.2±3.8 <sup>a</sup>	20.3±2.4 <sup>a</sup>	0.001	0.001	0.001
TAC (nmol/μL)	24.8±4.7	21.4±5.1 <sup>a</sup>	14.2±1.9 <sup>a</sup>	8.3±3.6 <sup>a</sup>	0.01	0.01	0.001
NO (nmol/dL)	12.7±0.42	15.8±0.87 <sup>a</sup>	24.8±0.97 <sup>a</sup>	34.5±1.3 <sup>a</sup>	0.01	0.001	0.001
sFas (ng/mL)	0.42±0.25	4.8±1.5 <sup>a</sup>	8.5±3.6 <sup>a</sup>	11.5±4.6 <sup>a</sup>	0.05	0.01	0.001
ssDNA (μg)	0.05×10 <sup>5</sup>	3.7×10 <sup>5</sup>	14.9×10 <sup>5</sup>	21.2×10 <sup>5</sup>	0.01	0.01	0.001

**Notes:** All values represent mean ± standard deviation; <sup>a</sup>significant difference from healthy controls at  $P \leq 0.05$ ; P<sub>1</sub>, comparison of ASC and fibrosis groups; P<sub>2</sub>, comparison of fibrosis and cirrhosis groups; P<sub>3</sub>, comparison of ASC and cirrhosis groups.

**Abbreviations:** 25(OH)D, 25-hydroxyvitamin D; CHC, chronic hepatitis C; ASC, asymptomatic CHC; TAC, total antioxidant capacity; sFas, soluble Fas; ssDNA, single-stranded DNA; ASC, asymptomatic CHC; NO, nitric oxide.



**Figure 1** Correlation between viral markers and serum 25(OH)D levels based on liver fibrosis. **Abbreviations:** 25(OH)D, 25-hydroxyvitamin D; HCV, hepatitis C virus; ASC, asymptomatic chronic HCV.

In the current study, based up on Metavir fibrosis-scoring system, only 70% of CHC patients were diagnosed with severe progression of fibrosis: they are classified into 25% with fibrosis (n=25) and 45% with cirrhosis (n=45), and only 30% (n=30) of patients were diagnosed as ASC carriers. There were significant changes in BMI, increases in serum levels of AST, ALT, and bilirubin, and decreases in albumin in CH patients compared with the control group. Our results are in line with other research, where the progression of hepatic fibrosis is significantly associated with higher rates of obesity-related factors, such as BMI.<sup>48</sup> Abnormal liver functions are expected to be associated with the progression of hepatic fibrosis. The obtained elevated serum levels of AST, ALT, and bilirubin and decreased albumin in CHC patients in our study were in accordance with most of the published data.<sup>21,49,50</sup> Other important inflammatory biomarkers released during CHC infection are AFP and TNF $\alpha$  levels in plasma. The elevated levels of AFP and TNF $\alpha$  in plasma of CHC patients compared to the controls were in agreement with other reports.<sup>51,52</sup>

The clinical data of our patients were correlative and supportive of previous reports that showed significant decreases in platelet counts and increases in glucose and HOMA-IR compared to controls.<sup>53,54</sup> Also, the results of our study showed that activity and progression of chronic liver disease measured by Metavir fibrosis stages (F0–F4), necroinflammatory activity grades (A0–A3), and viral load (38.7 $\pm$ 6.2) were similar to those previously reported.<sup>55,56</sup>

Significant 25(OH)D deficits have been reported in almost half of the healthy population of developed countries.<sup>51</sup> Besides its effects in bone health, 25(OH)D deficiency has been reported in the progression of many diseases, such as several types of cancers and cardiovascular and infection-related diseases, including CHC.<sup>57–60</sup>

Correlations between 25(OH)D levels and the progression of liver fibrosis were estimated in CHC patients under this study. Lower levels of 25(OH)D were significantly reported in CHC patients with fibrosis, cirrhosis, and ASC compared with control subjects. The data showed that 25(OH)D deficits correlated negatively with abnormal

**Table 4** Correlation coefficients (*r*) of apoptosis-related markers ssDNA and sFas with other parameters in geriatric patients with CHC (n=100)

Groups/ parameters	Apoptotic markers					
	ASC (n=30)		Fibrosis (n=25)		Cirrhosis (n=45)	
	sFas	ssDNA	sFas	ssDNA	sFas	ssDNA
HCV RNA (IU/mL)	0.15*	0.35*	0.15**	0.35**	0.65***	0.40***
Viral load	0.23*	0.18*	0.12**	0.64**	0.88***	0.31***
AFP (ng/mL)	0.45*	0.35*	0.24**	0.76**	0.61***	0.39***
TNF $\alpha$ (pg/mL)	0.38*	0.18*	0.26**	0.36**	0.58***	0.58***
TAC (nmol/ $\mu$ L)	0.31*	0.51*	0.48**	0.64**	0.71***	0.67***
NO (nmol/dL)	0.37*	0.35*	0.54**	0.53**	0.56***	0.62***

**Notes:** \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001.

**Abbreviations:** ssDNA, single-stranded DNA; sFas, soluble Fas; CHC, chronic hepatitis C; ASC, asymptomatic CHC; HCV, hepatitis C virus; TAC, total antioxidant capacity; AFP, alpha fetoprotein; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; NO, nitric oxide.

**Table 5** Correlation coefficients (*r*) of 25(OH)D deficiency with other parameters in geriatric patients with CHC (n=100)

Groups/ parameters	25(OH)D deficiency (nmol/L)		
	ASC (n=30)	Fibrosis (n=25)	Cirrhosis (n=45)
Age	-0.22*	-0.84*	-0.93*
Sex	-0.18*	-0.28*	-0.31**
Liver function	-0.32**	-0.43**	-0.71***
BMI	-0.28**	-0.23**	-0.51***
HOMA-IR	-0.48**	-0.63**	-0.71***
HCV RNA (IU/mL)	-0.35**	-0.198**	-0.510***
Viral load	-0.18**	-0.214**	-0.478***
AFP (ng/mL)	0.35**	0.251**	0.612***
TNF $\alpha$ (pg/mL)	0.18**	0.243**	0.478***
TAC (nmol/ $\mu$ L)	0.51**	0.78**	0.681***
NO (nmol/dL)	0.35**	0.514**	0.156***
sFas (ng/mL)	0.231**	0.452**	0.714***
ssDNA ( $\mu$ /g)	0.583**	0.463**	0.245***

**Notes:** \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Abbreviations:** 25(OH)D, 25-hydroxyvitamin D; CHC, chronic hepatitis C; ASC, asymptomatic CHC; BMI, body mass index; HOMA, homeostatic model assessment; IR, insulin resistance; HCV, hepatitis C virus; TAC, total antioxidant capacity; sFas, soluble Fas; ssDNA, single-stranded DNA; AFP, alpha fetoprotein; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; NO, nitric oxide.

liver function, viral load, and HCV RNA expression. The deficiency in 25(OH)D levels among our patients was shown to be associated with liver severity, as reported in most of the literature, whereas vitamin D deficiency has been estimated among CHC cases with higher rates of mortality,<sup>61–63</sup> portal hypertension,<sup>64</sup> and fibrosis severity.<sup>65</sup> 25(OH)D deficits were proposed to be linked with poor hepatic hydroxylation of vitamin D to 25(OH)D or calcidiol,<sup>47</sup> lower albumin, and decreased expression of DBP, which is synthesized mainly in the liver, and in normal liver cells ~88% of serum 25(OH)D is attached to a DBP.<sup>66</sup> It was reported that only 5% of DBP-binding sites were required for the binding of vitamin D metabolites,<sup>67</sup> and this confirms that liver dysfunction should be severe enough to decrease DBP levels and ultimately produce vitamin D deficiency.<sup>68</sup> Therefore, these previous reports explained the variability in deficiency rates of vitamin D levels among our CHC patients, especially those with cirrhosis.

Also, other studies also showed a close association between 25(OH)D deficiencies, degree of fibrosis and necrosis in CHC patients, and decrease in sustained viral response factor toward HCV viral treatments, especially IFN/ribavirin (RBV)-based therapies.<sup>5,21,50,69</sup>

Vitamin D defects in CHC patients with fibrosis were shown to be inversely correlated with age, sex, HOMA-IR, and obesity (BMI). Negative correlations between 25(OH)D levels and obesity, glucose intolerance, IR, and BMI have been reported in many diseases, especially liver disorders.<sup>70,71</sup>

In addition, previous studies have shown an increase in the accumulation of vitamin D in adipose tissue of obese patients, which might relate to vitamin D deficiency.<sup>72,73</sup> In contrary, although vitamin D levels are inversely correlated with fibrosis and necroinflammation of liver tissues, this correlation is independent of age, sex, BMI, HOMA-IR score, and presence of metabolic syndrome,<sup>74–76</sup> whereas normal vitamin D levels have been shown to enhance HCV response to IFN and RBV therapy<sup>77</sup> via improving sensitivity to insulin<sup>78</sup> and prevent the development and progression of fatty liver by modulation of lipid metabolism.<sup>79</sup>

In the present study, the deficiency in vitamin D levels also showed significant associations with inflammation and fibrosis among CHC patients. There were positive correlations among vitamin D defects and the release of both AFP and TNF $\alpha$  as inflammatory markers in patients with severe fibrosis scores. Previous evidence that matches our results suggests that during inflammatory diseases of the liver, the production of active vitamin D is affected due to significant decreases in the expression of vitamin D receptors. These receptors were significantly expressed in tissue macrophages, whereas it comprises of ~90% in the liver, non-parenchymal cells, and biliary epithelial cells.<sup>80,81</sup> Vitamin D in its active form is involved in the decrease of inflammation.<sup>21,76,82</sup> Also, it has been reported that excessive inflammatory response was regulated by proinflammatory signals present in monocytes and macrophages. These signals may regulate the local metabolism of vitamin D through autoexpression of CYP27B1 and the local production of 1 $\alpha$ , 25(OH)<sub>2</sub>D. It has been proposed that antifibrotic and anti-inflammatory activity of vitamin D decreases the persistence of HCV against IFN/RBV therapy. This occurs via minimizing the induction of proinflammatory cytokines.<sup>20,83</sup>

In various liver diseases, pathogenesis and progression attributed to initiation of higher amounts of oxidative stress free radicals, such as ROS. Mitochondria, cytochrome P450 enzymes, Kupffer cells, and neutrophils were shown to be the main sources of ROS.<sup>9,84</sup>

The excessive production of ROS produces significant damage to cellular proteins, the cell wall, and DNA. These harmful effects can be nullified with both enzymatic (super-oxide dismutase, catalase, glutathione peroxidase), and non-enzymatic antioxidants, such as vitamins (A, C, E, D), and reduced glutathione.<sup>85</sup> Therefore, CHC patients included in this study were subjected to estimation of the nitric oxide free radical (NO) and TAC as markers of oxidative stress.

The consequence increase in the levels of NO and decrease in the levels of TAC activity among patients with

cirrhosis and fibrosis were reported as Oxidative stress parameters compared with ASC subjects. These data were in line with other studies that reported the role of oxidative stress in potentiating the pathogenesis and progression of liver diseases.<sup>9,20,78–84,86,87</sup> Also, the decrease in vitamin D levels of the studied subjects was positively correlated with changes in both NO and TAC in CHC patients with varying pathological lesions.

Previous studies have reported that low vitamin D levels were estimated in >92% of HCV patients, with 25% of these patients suffering from severe vitamin D deficiency.<sup>21,88</sup> It was proposed that HCV infection may affect direct or indirect 25-hydroxylation of vitamin D through cytokine induction or oxidative stress,<sup>89</sup> or suppression of 25(OH)D levels via a disruption in lipid metabolism.<sup>90</sup>

On a molecular basis, little is known about events leading to cellular damage and progression of liver diseases among CHC patients; however, host cells and viral and environmental factors may have a significant role in the pathogenesis of liver. Apoptosis is one of the molecular processes that occurs in both normal and pathological organs to maintain tissue development and homeostasis.<sup>91</sup> Previous research has reported that hepatocyte apoptosis plays a role in liver fibrogenesis, persistent liver inflammation, and subsequent severe pathogenesis of the liver among patients with CHC.<sup>92–94</sup>

In the present study, higher rates of liver cell apoptosis were reported in CHC patients. There was a significant increase in the expression levels of ssDNA and sFas as apoptotic markers in patients with cirrhosis and fibrosis compared with ASC and healthy control subjects. The data were in line with many previous studies that reported significant increases in levels of ssDNA and sFas and their association with fibrosis.<sup>95–97</sup> Similarly, a significant positive correlation was reported between increased ssDNA, sFas, and fibrosis levels in CHC patients,<sup>95,98</sup> which suggests that increased levels of these markers may reflect the degree of liver fibrosis.

The overexpression of apoptotic markers was shown to be positively correlated with oxidative stress markers: NO, TAC, inflammatory cytokines, TNF $\alpha$ , AFP, viral load, and HCV RNA. The data were matched with previous reports that supported the association of apoptosis with the expression of oxidative stress,<sup>87</sup> inflammatory cytokines,<sup>93</sup> and HCV-infection parameters.<sup>99–101</sup> Also, deficits in vitamin D levels showed positive significant correlation with apoptosis among our CHC patients under investigation. Our data are in agreement with previous studies supporting the association of vitamin D-deficient status and excessive expression of Fas and ssDNA apoptotic markers,<sup>17,95,102</sup> whereas long-term vitamin D

deficiency can activate chronic inflammation and subsequently higher oxidative stress that can induce apoptosis.

Finally, HCV genotype 4 is the most prevalent genotype in North Africa, the Middle East, and central and east sub-Saharan Africa, and prevalence has been increasing in Europe.<sup>103–106</sup> Our study is considered the first to evaluate the potential role of 25(OH)D status in the progression of liver fibrosis via apoptotic and oxidative mechanisms in geriatric patients with HCV genotype 4.

## Conclusion

The data showed that vitamin D status was significantly correlated with the pathogenesis and fibrogenesis of the liver in geriatric patients infected with HCV genotype 4. The deficiency in vitamin D levels was shown to have severe pathological effects on the liver via apoptosis, oxidative stress, and inflammatory mechanistic pathways. The data suggest that adequate vitamin D levels be recommended for good response to treatment strategies, especially in older CHC patients.

## Acknowledgment

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project RGP-VPP-240.

## Disclosure

The authors report no conflicts of interest in this work.

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