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THE CORRELATION OF VITAMIN D LEVELS WITH VIROLOGICAL PARAMETERS IN PATIENTS WITH TREATMENT NAIVE CHRONIC HEPATITIS B INFECTION

General Medicine

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ABSTRACT

Hepatitis B virus infection is associated with a significant risk of developing cirrhosis, and hepatocellular carcinoma. Most patients need life-long therapy with Nucleotide Analogs.The development of novel immunomodulatory approaches to control HBV infection, appears to be highly relevant. Cholecalciferol is the precursor of the bioactive calcitriol serves as an important modulator of numerous signaling pathways related to both innate and adaptive immunity.Therapeutic value of the immunomodulatory effect of vitamin D has been proven.

AIMS AND OBJECTIVES: The aim of this study is to evaluate the clinical utility of vitamin D levels in management of treatment naive chronic HBV infection.To correlate between serum vitamin D levels and HBV DNA viral load in patients with chronic hepatitis B infection.

MATERIALS AND METHODS: Study was conducted in the department of Gastroenterology, Yashoda Hospital, Hyderabad from November 2014 to March 2016. A total of 55 treatment naive chronic hepatitis B (CHB) patients were included in this study.Data was collected prospectively from consecutive patients.Blood samples were taken for vitamin D levels, quantitative HBV DNA, HBeAg for CHB patients.Quantitative HBV DNA levels were done by ABI real time PCR.25-Hydroxy Vitamin D 25(OH)D levels were done by EIA.

RESULTS: A total of 55 patients, 36 were males and 19 were females with mean age of 43 (±14) years . The Mean serum vitamin D concentrations of entire cohort was 20.4±15.7) ng/ml. 17 (31%) had severe vitamin D deficiency [25(OH)D <10ng/ml].Patients having HBV DNA levels > 20,000 IU/ml had significantly low serum vitamin D levels compared to patients with serum HBV DNA levels <20,000IU/ml ($p < 0.0001$).The mean serum vitamin D level in HBeAg positive patients was 6.65(2.63) and in HBeAg negative patients was 33.57(14.85)($p<0.0003$).The mean vitamin D serum concentrations in autumn-winter and spring-summer months were 12.4 ± 4.2 and 20.6 ± 2.2 ng/ml, respectively ($p < 0.0001$).

CONCLUSION:

1. HBV DNA viral load appears to be the strongest determinant of low 25(OH)D serum levels in treatment naïve CHB patients.
2. HBV DNA viral load appear to have an inverse relationship with serum vitamin D levels.
3. HBeAg positive CHB patients appear to be more deficient in serum vitamin D as compared to HBeAg negative patients.
4. Serum Vitamin D levels appears to have significant seasonal fluctuations.

KEYWORDS

Hepatitis B Virus,Hepatitis B Virus (HBV) DNA ,Vitamin D

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health problem worldwide. Hepatitis B is an infectious disease, affecting an estimated 350 million chronically infected patients[1,2]. Despite the availability of potent vaccines, infection with hepatitis B virus still represents one of the most significant infectious diseases worldwide[3]. Hepatitis B virus is associated with a significant risk of developing severe liver disease including liver failure, cirrhosis, and hepatocellular carcinoma (HCC). HBV is not directly cytopathogenic towards hepatocytes, the interaction between the virus and the host immune response plays a significant role in the pathogenesis of liver disorders[4-6]. These immune responses occur as complex interferences between the inborn immune and adaptive immune response[7].

The natural course of chronic HBV infection has five phases including the immune tolerant phase, immune reactive hepatitis B e antigen (HBeAg) positive phase, inactive HBV carrier state, HBeAg-negative chronic HBV and hepatitis B surface antigen (HBsAg) negative phase[8]. Patients with chronic hepatitis B who have HBV DNA levels >2,000 IU/mL, elevated serum alanine aminotransferase (ALT) levels, and at least moderate fibrosis or necroinflammation in liver biopsies require antiviral therapy to avoid progression of liver diseases to cirrhosis and its complications[8-10].it was estimated that most patients will depend on life-long therapy with Nucleotide Analogs(NAs)[9-11]. The development of novel therapeutic strategies, especially of novel immunomodulatory approaches to control HBV infection, appears to be highly relevant.

Cholecalciferol is the precursor of the bioactive vitamin D metabolite, calcitriol[12]. Nutritional sources of cholecalciferol are rare and its largest proportion in humans is synthesized in the skin during exposure to ultraviolet light[13]. The bioactive vitamin D metabolite, $1,25(OH)_2D_3$, which is also called calcitriol, exerts its biological functions predominantly by signaling through a nuclear vitamin D receptor (VDR), which serves as a ligand-activated transcription factor[12]. Clinical assays to quantify calcitriol are generally characterized by poor reliability[13].$25(OH)D_3$, is usually measured in clinical routine to assess a patient's vitamin D status[13]. By induction or repression of expression of hundreds of genes, calcitriol serves as an important modulator of numerous signaling pathways related to both innate and adaptive immunity[13,14-16].In patients with tuberculosis, a therapeutic value of the immunomodulatory effect of vitamin D has already been proven in randomized, controlled clinical trials[17,18]. Importantly, vitamin D supplementation in these studies resulted in an increased activity of intrinsic interferon-alpha (IFN-α) signaling.[19]

The main purpose of this study is to evaluate the correlation of vitamin D levels with virological parameters in patients with treatment naive chronic HBV infection.

AIMS AND OBJECTIVES

The aim of this study is to evaluate the clinical utility of vitamin D levels in management of treatment naive chronic HBV infection.

To correlate between serum vitamin D levels and HBV DNA viral load in patients with chronic hepatitis B infection.

MATERIALS AND METHODS

Study was conducted in the department of Gastroenterology, Yashoda Hospital, Hyderabad from November 2014 t0 March 2016. Informed consent of the study participants was obtained in all cases. The study had approval of local Ethical Committee. It was a prospective study. We screened 75 Treatment naive chronic hepatitis B (CHB) patients for the study and a total of 55 patients were included in this study

INCLUSION AND EXCLUSION CRITERIA:

Inclusion Criteria:

1. Treatment naïve Chronic HBV infected patients (CHB) defined as detectable hepatitis B surface antigen (HBsAg) , HBV DNA ≥ 6 months
2. Age ≥18 years

Exclusion Criteria:

1. Coinfection with HCV/HBV, human immunodeficiency virus (HIV).
2. Malignancy including HCC.
3. Chronic renal failure (serum creatinine > 1.25mg/dl).
4. Thyroid disorders.
5. History of calcium or Vitamin supplements within previous 3 months.

METHODOLOGY:

Data was collected prospectively from consecutive patients from both outdoor and indoor patients based on clinical interview and review of records.Detailed history taken and Patients were subjected to a detailed general and systemic examination. Blood samples were obtained for complete blood count, renal function test, liver function test, Thyroid function tests, serum calcium levels etc. Blood samples were taken for vitamin D levels, quantitative HBV DNA, HBeAg for CHB patients.

Quantitative HBV DNA levels were done by ABI real time PCR using Taqman Chemistry**.** The viral load is expressed both as copies/mL as well as IU/mL (VIRAL LOAD 1IU = ~5.82 COPIES), linearity (17.1 – 34364262 IU/mL or 100 – 200000000 copies /mL). Hepatitis B Virus 'e' Antigen was done by enzyme immunoassay test (EIA) and was reported as 'Positive or Negative with a cut off value of 0.100.

25-Hydroxy Vitamin D 25(OH)D levels were done by EIA and values were measured in ng/L. Patients were classified into three groups as per their vitamin D levels as vitamin D deficient (≤10 ng/ml),insufficient (11-20 ng/ml) and optimal (>20 ng/ml).

STATISTICAL METHODS:

HBV DNA and vitamin D levels are numeric variables, so the mean and standard deviation was calculated. After analyzing normal or non-normal distribution of the continuous variables, continuous data was examined using the student t test (if normally distributed), Mann–Whitney test (if non-normally distributed), and categorical variables were examined by chi square test. The relationship and comparison between viral loads and vitamin D levels was assessed using Pearson correlation coefficient.

Associations between dichotomic (e.g., hepatitis B early antigen [HBeAg]-positive versus negative) was assessed by logistic regression models and linear (e.g., HBV DNA serum concentration) variables and $25(OH)D_3$ serum levels were assessed by linear regression models. After univariate analyses, multivariate analyses was performed for significant associations. Multivariate models was obtained by backward selection, using a *P* value >0.15 for removal from the model. Group differences were assessed by means of chi-square contingency tables or Wilcoxon-Mann-Whitney's U tests, as appropriate.

RESULTS

Table1: Demographic, clinical and biochemical features of HBV patients

Parameters	Total Patients (N=55)
Age(yrs)	43.7 (14)
Sex(M/F)	36/19
BMI(kg/m^2)	23.8(1.7)
Hb(g/dl)	13.3 (1.7)
TLC (/µl)	6.8 (2.3)
Platelet(10^9//L)	2.3 (0.8)
Bilirubin (mg/dl)	0.9 (0.5)
SGOT (U/L)	41.8 (24.6)
SGPT (U/L	43.1 (33)
ALP (U/L)	91.9 (18.2)
Total Protein (g/dl)	6.9 (0.5)
Albumin (g/dl)	3.7 (0.4)
Prothrombin Time (seconds)	14.8 (1.6)
Serum Creatinine (mg/dl)	1.0 (1.1)
Serum Calcium (mg/dl)	9.6 (0.7)
TSH (µIU/ml)	2.6 (1.4)
25(OH)D (ng/ml)	20.4 (15.7)
HBV DNA log10(IU/ml)	3.04(2.08)
HBeAg (Positive/Negative)	18/37

Values are shown as Mean (SD)

A total off 55 patients were selected in hepatitis B group according to the inclusion criteria. Out of 55 patients 36 were males and 19 were females with mean age of 43 (±14) years . The majority of the patients were HBeAg negative. Mean serum vitamin D concentrations of entire cohort was 20.4±15.7) ng/ml.

Table 2: Demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to 25(OH)D levels

25(OH)D (ng/ml)				
	>20	10-20	<10	p value
Number (%)	12 (21.8)	25(45.4)	17 (30.9)	
25(OH)D3 (ng/ml)	40.3(11.4)	15.9(3)	6.4(1.8)	0.0001
Age(yrs)	41(16)	40 (8)	45 (15)	0.405
Sex (M/F)	22/12	9/4	5/3	
BMI(kg/m^2)	23.2(3.2)	23.9(2.4)	23.6(2.5)	0.988
Hb(g/dl)	13.8(1.4)	12.7(1.6)	12.9(1.4)	0.290
TLC (/µl)	6.9(2.3)	6.4(1.4)	6.9(3.3)	0.814
Platelet(10^9//L	2.4(0.9)	2.1(0.4)	2.4(1.5)	0.488
Bilirubin (mg/dl)	0.9(0.5)	0.7(0.3)	1.0(0.8)	0.512
SGOT (U/L)	37.5(16.8)	35.6(16.3)	33.3(11.4)	0.216
SGPT (U/L	36.7(36.4)	48(29)	59.5(16.9)	0.177
ALP (U/L)	90.9(20.3)	93.1(13.4)	94.3(16.8)	0.864
Total Protein (g/dl)	7.0(0.6)	6.9(0.3)	6.8(0 .4)	0.141
Albumin (g/dl)	3.7(0.4)	3.8(0 .3)	3.3(0.4)	0.021
Prothrombin Time (seconds)	14.4(1.0)	14.6(0 .9)	15.6(3.1)	0.022
Serum Creatinine (mg/dl)	0.9(0.1)	1(0.2)	0.8(0 .2)	0.184
S. Calcium (mg/dl)	9.6(0 .6)	9.6(0 .7)	9.6(1.1)	0.986
TSH (µIU/ml)	2.5(1.4)	2.4(1.0)	2.9(2.1)	0.784
HBV DNA Log10 (IU/mL)	1.07	2.37(1.07)	5.54(1.55)	0.0001
HBeAg (Positive/Negative)	0/13	01/24	17/0	<0.0001

Values are shown as Mean (SD)

The comparison of demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to 25(OH)D levels were done. No significant differences were observed in any of these parameters in relation to 25(OH)D levels except for HBV DNA and HBeAg.

Of the 55 patients of the entire cohort, 17 (31%) had severe vitamin D deficiency [25(OH)D <10ng/ml], 25 (47%) had insufficient vitamin D levels [25(OH)D ≥10 and <20ng/ml] and 12 (22%) had optimal vitamin D levels [25(OH)D ≥ 20ng/ml] (p <0.0001). All patients with vitamin D levels ≥20ng/ml were HBeAg negative, whereas all patients with vitamin D levels <20ng/ml were HBeAg positive and this difference was statistically significant (p<0.0001). Patients with vitamin D levels below 20 ng/m had significantly high viral loads as compared to patients with vitamin D levels ≥ 20ng/ml (p<0.0001).Hence vitamin D deficiency and insufficiency was highly prevalent in CHB patients.

The comparison of demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to HBV DNA levels (**Table 3**) were also done. No significant differences were observed in any of these parameters in relation to HBV DNA levels except for 25(OH)D and HBeAg. Vitamin D concentrations were significantly low in patients who had high viral loads (p<0.0001). Similarly HBeAg positive patients had low serum vitamin D levels as compared to HBeAg negative patients (p<0.0001).

Table 3: Demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to HBV DNA levels

Number (%)	23(41.8)	12(21.8)	09(16.3)	11(20)	
Age(yrs)	44.5(13)	37.4(15)	40.3(7.0)	41 (11.8)	0.459
Sex(M/F)	14/9	07/05	08/01	07/04	
BMI(kg/m2)	25.0(1.1)	24.8(1.2)	25.1(0.9)	24.9(1.0)	0.908
HB(g/dl)	13(1.7)	13.8(1.8)	14 (1.5)	12 (1.8)	0.068
TLC (/µl)	6.5(2.3)	8 (1.9)	6.3 (1.4)	6.6 (2.8)	0.226
Platelet(109//L)	2.2(0.7)	2.6 (0.8)	2.1 (0.4)	2.4 (1.2)	0.448
Bilirubin (mg/dl)	0.8(0.5)	0.9 (0.4)	0.8 (0.3)	0.9 (0.7)	0.912
SGOT (U/L)	31(15.5)	46.5(23.4	40.8(17.5	45.9(17.3)	0.178

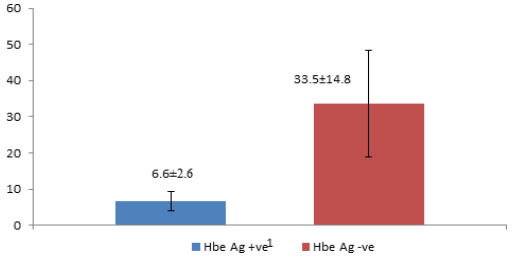
SGPT (U/L	33.7(10.9	32.8(15.6	51.4(27.2	57.2(18.7)	0.011
ALP (U/L)	93.3(19.8	81.1(13.7	97.2(7.7)	90.4 (18)	0.013
Total Protein (g/dl)	7.1(0 .7)	6.9 (0.4)	6.9 (0.3)	6.6 (0.4)	0.105
Albumin (g/dl)	3.7(0.4)	3.7 (0.3)	3.7 (0.2)	3.5 (0.5)	0.491
Prothrombin Time (seconds)	14.3(0 .9)	14.5 (1)	14.8(0.8)	15.8 (3)	0.078
Serum Creatinine (mg/dl)	0.9(0 .1)	0.9 (0.1)	1.0 (0.1)	0.8 (0.1)	0.110
Serum Calcium (mg/dl)	9.6(0.6)	9.7 (0.6)	9.5 (0.8)	9.7 (0.9)	0.901
TSH (µIU/ml)	2.5(1.4)	3 (1.3)	1.8 (0.9)	2.8 (1.9)	0.271
HBV DNA (IU/mL)	12(0)	339.4 (338)	6275 (3866.3)	28677906 (39888698)	0.0001
HBeAg Positive/Negative	0/19	0/10	02/05	16/03	0.0001
25(OH)D (ng/ml)	44.8(11.4	26.7(4.9)	20.3 (9)	8.2 (3.7)	0.0001

Values are shown as Mean (SD)

Patients having HBV DNA levels > 20,000 IU/ml had significantly low serum vitamin D levels compared to patients with serum HBV DNA levels <20,000IU/ml (p <0.0001).

Table 4: Comparison of 25(OH)D levels according to HBeAg

HBeAg	25(OH)D3	P value
Positive (18/55)	6.65(2.36)	0.0003
Negative (37/55)	33.57(14.85)	



Out of 55 cases 18 patients were HBeAg positive and 37 patients were HBeAg negative. The mean serum vitamin D level in HBeAg positive patients was 6.65(2.63) and in HBeAg negative patients was 33.57 (14.85), which was statistically significant (p<0.0003).

To evaluate the relationship between 25(OH)D and HBV DNA serum levels, we stratified the patients according to HBV DNA serum levels <2,000 IU/ml versus >2,000IU/ml (Table 5).Patients with HBV DNA viral load below this threshold, which is generally considered as relevant for clinical decision making, had substantially higher means 25(OH)D serum levels, compared to patients with HBV DNA ≥2,000IU/ml (18 versus 39 ng/ml, respectively; p<0.001).

Table 5: Comparison of 25(OH)D levels according to HBV DNA (IU/ml)

HBV DNA (IU/ml)	25(OH)D	P value
<2000 (35/55)	38.76(12.87)	0.0001
>2000 (20/55)	13.71(8.93)	

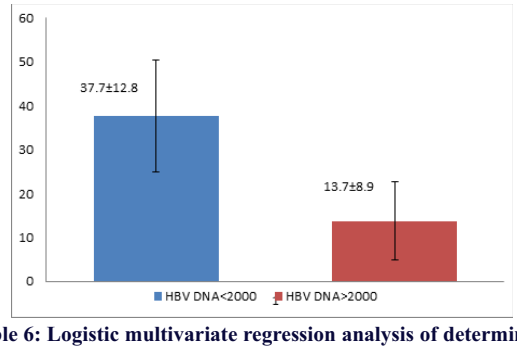


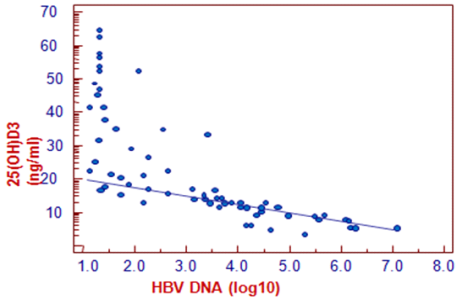
Table 6: Logistic multivariate regression analysis of determinant factors associated with 25(OH)D in CHB patients.

Age(yrs)	0.18	0.09
BMI (kg/m^2)	0.29	0.78
HB (g/dl)	0.07	0.17
Platelets(10^9/l)	0.28	0.27
Creat. (mg/dl)	0.47	0.99
Bilirubin (mg/dl)	0.25	0.56
AST (mg/dl)	0.03	0.99
ALT(mg/dl)	0.03	0.75
Albumin (g/dl)	0.14	0.85
Prothrombin Time (seconds)	0.37	0.88
TSH (µIU/ml)	0.24	0.52
HBeAg (Positive/ Negative)	0.0001	0.614

In both univariate and multivariate analyses, HBV DNA was the major determinant factor of low 25(OH)D levels , p < 0.0001.

Table 7: Pearson Correlation between HBV DNA and 25(OH)D levels:

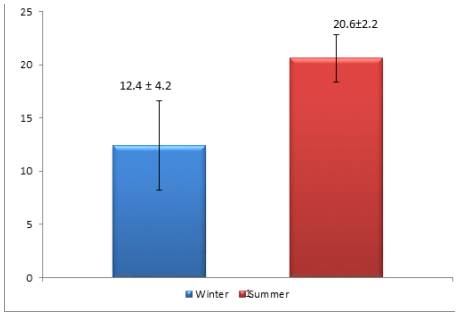
Correlation coefficient r	-0.676
Significance level	P=0.0001



25(OH)D and HBV DNA serum levels showed a significant inverse correlation, r= -0.676, (p=0.0001).

To assess the relationship between 25(OH)D serum level and season, the study period was divided into autumn-winter months (November-April; n = 25/55, 45%) and spring-summer months (May-October; n=30/55, 55%) depending on the date of vitamin D testing. The mean vitamin D serum concentrations in autumn-winter and spring-summer months were 12.4 ± 4.2 and 20.6 ± 2.2 ng/ml, respectively. The difference between seasons was statistically significant (p < 0.0001).

Bar chart depicting the seasonal variation of vitamin D levels:



DISCUSSION

Recently, it has been recognized that vitamin D has other functions in addition to its role in bone metabolism[20]. It has been demonstrated that vitamin D deficiency may play a role in the development of autoimmune diseases, inflammatory bowel disease, rheumatoid arthritis, psoriasis, multiple sclerosis, diabetes, certain cancer types, cardiac failure, stroke and infectious diseases such as tuberculosis and pneumonia, and that vitamin D supplementation is efficacious in these patients[21-25].

Chronicity of hepatitis B infection is also influenced by mutations in the vitamin D receptor gene, with polymorphisms being associated with higher viral load and increased disease progression and severity. Of note, the t-allele is associated with enhanced Th1 cellular immunity and promotes more efficient clearance of several viral infections, including hepatitis B and dengue virus[26,27].

The possible causal relationship between vitamin D metabolism and HBV replication, which needs to be proven by future studies, may offer attractive therapeutic opportunities for treatment of CHB. Very few studies have demonstrated an association between serum vitamin D

levels and viral loads. The present study demonstrates a profound association between higher levels of HBV replication and low 25(OH)D serum levels in CHB patients.

Comparison between few recently published studies and our study is depicted in following tabular form.

Parameters	Farnik *et al*[28]	Demir *et al*[29]	Mohammed Amin Mohammed *et al*[30]	Our study
Number	203	35	75	55
Age in years (Mean)	39	32.5	49.7	43.7
Male/Female	107/96	22/13	39/36	36/19
Vitamin D Levels Ng/ml (Mean)	14.4	7.65	13.9	20.4
HBV DNA Levels (Mean)	3.47 log10 IU/ml	-	882.04 Iu/ml x103	3.04 log 10 IU/ml
HBeAg Positive/Negative	177/28	-	-	18/37
BMI (kg/m2) (Mean)	22	22.9	22.3	23.8
ALT (U/L) (Mean)	40	31.25	30.07	43.8
AST(U/L) (Mean)	-	29.17	39.1	41.8
TSH (μIU/ml) (Mean)	-	1.35	1.46	2.6
Creatinine (mg/dl) (Mean)	-	0.89	1.09	1.0

In our study majority population was male (65.5%) and the mean age of population under study was 43.70 years. The mean serum vitamin D levels in our study population was 20.4 ± 15.7 ng/ml. Majority of our patients were HBeAg positive(67%).The mean HBV DNA levels of the whole cohort was 3.04 ± 2.08 log10 IU/ml which is comparable to other studies.[28,31].

Of the entire cohort, 31% had severe vitamin D deficiency [25(OH)D <10ng/ml], 47% had insufficient vitamin D levels [25(OH)D ≥10 and <20ng/ml] and 22% had optimal vitamin D levels [25(OH)D ≥ 20ng/ml] (p <0.0001) which is comparable with the other studies.

All patients with vitamin D levels ≥20ng/ml were HBeAg negative, whereas all patients with vitamin D levels <20ng/ml were HBeAg positive and this difference was statistically significant (p<0.0001) which is in contrast to the study by Farnik .

In our cohort, HBV DNA viral load appears to be the strongest determinant of low 25(OH)D serum levels which is comparable with the study by Farnik et al[28]. Patients with HBV DNA viral load <2000IU/ml, which is generally considered as relevant for clinical decision making, had substantially higher mean 25(OH)D serum levels, compared to patients with HBV DNA >2,000 IU/mL (39 versus 14 ng/mL, respectively P<0.0001) which is comparable with the study by Farnik.

Patients with vitamin D levels below 20 ng/ml had significantly high viral loads as compared to patients with vitamin D levels ≥ 20ng/ml (p<0.0001).Hence vitamin D deficiency and insufficiency was highly prevalent in CHB patients. Moreover we observed a significant inverse relationship between serum vitamin D levels and HBV DNA levels which is also comparable to the study by Demir at al[29] and Farnik et al.

Infection by hepatitis B virus causes complicated immunological, biochemical and histological changes in host immune response which can be specific or non specific. The non specific response occurs via cytokines or other substance. Vitamin is known to suppress pro-inflammatory cytokines and increase interleukin 10[32].Thus it could be suggested that vitamin D deficiency may be related to increased viral replication and viral load.

In our study inversed seasonal fluctuations of 25(OH)D and HBV DNA serum levels were seen as shown by other studies.

The high prevalence of vitamin D deficiency in patients with chronic liver illness occurs regardless of disease etiology. It was recently suggested that vitamin D may impact both clinical outcomes and clinical response in patients with CHC. Several in vitro studies have demonstrated that vitamin D inhibits HCV replication in a dose dependent manner[33,34]. There are conflicting data in the literature regarding the relationship between baseline 25OHD levels and the attainment of SVR. A few studies did not found any association between vitamin D deficiency and the rates of viral clearance[35]. A meta-analysis by Villar LM et al demonstrated high prevalence of vitamin D deficiency and high SVR in individuals with higher serum vitamin D levels or receiving vitamin D supplementation.[36]

CONCLUSION:

1. HBV DNA viral load appears to be the strongest determinant of low 25(OH)D serum level in treatment naïve CHB patients.
2. HBV DNA viral load appear to have an inverse relationship with serum vitamin D level.
3. HBeAg positive CHB patients appear to be more deficient in serum vitamin D as compared to HBeAg negative patients.
4. Serum Vitamin D levels appears to have significant seasonal fluctuations.

ABBREVIATIONS

HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
CHB	Chronic Hepatitis B
HBeAg	Hepatitis B e Antigen
HBsAg	Hepatitis B Surface Antigen
ALT	Alanine aminotransferase
SVR	Sustained Virological Response
25(OH)D	25-Hydroxy Vitamin D
VDR	Vitamin D Receptor
CLIA	Chemiluminescence Immunoassay
RBV	Ribavirin
PEG-IFNα	Pegylated Interferon α
NAs	Nucleotide Analogs
PTH	Parathyroid Hormone

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