

Title:

**The causal relationship between serum vitamin D level and hepatitis B virus replication in patients with chronic hepatitis B virus infection**

**NCT 03068767**

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**IRB approval letter (as attached)**

## **Abstract**

**Background and Aims:** Vitamin D deficiency is common in patients with chronic hepatitis B virus (HBV) infection. Previous study showed an inverse association between vitamin D and HBV DNA levels. However, the causal relationship between vitamin D and HBV replication remains inconclusive.

**Methods:** HBV carriers receiving regular 6-month surveillance without current antiviral treatment or cirrhosis were invited to participate into this trial. The markers of HBV replication included serum HBV DNA and quantitative HBsAg (qHBsAg) levels. Those with undetectable HBV DNA or sufficient vitamin D levels, cancer or electrolyte imbalance were excluded. The eligible subjects were randomized to receive either vitamin D supplement 2000 IU per day for 2 months (vitamin D group) or none (control group). Levels of vitamin D, HBV DNA and qHBsAg before and after 2 months of treatment or follow-up were compared between these two groups. The primary endpoint was to identify any difference between two groups.

## **Introduction**

Hepatitis B virus (HBV) infection is a global health problem, especially in Taiwan.

The clinical manifestations of HBV infection include acute/fulminant hepatitis or various forms of chronic infection. Chronic HBV infection may lead to inactive carrier, chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma (HCC).<sup>1-5</sup> Low vitamin D is frequently found in patients with chronic hepatitis B (CHB) than healthy controls.<sup>6</sup> Furthermore, several cross-sectional studies showed an inverse association between serum vitamin D level and HBV DNA level.<sup>7-10</sup> However, the causal relationship cannot be confirmed from these studies.

Cholecalciferol is the precursor of the bioactive vitamin D. It is rare in natural foods and the major source is synthesized in the skin during exposure to sunlight.<sup>11</sup> It is hydroxylated in liver to calcidiol, which is released into the plasma and exerts its biological functions by signaling through a nuclear vitamin D receptor. Therefore, liver has a close relationship with the synthesis of vitamin D. Vitamin D plays a crucial role in the bone metabolism and affect immune function not only in innate but also in adaptive immunity.<sup>12,13</sup> In clinical practice, low vitamin D is associated with autoimmune, infective disease, and the development of malignancy.<sup>14</sup>

Vitamin D has been reported to be an important immune modulator of hepatitis C virus (HCV) infection and metabolic liver disease.<sup>15-20</sup> Regarding HBV-infected patients, the relationship among vitamin D level, HBV viral load and liver dysfunction remains largely unclear. To clarify the causal relationship, we conducted a randomized controlled trial of providing vitamin D supplement to HBV patients with insufficient vitamin D (<30 ng/mL) level to investigate the effect of vitamin D level on HBV replication in terms of HBV DNA and qHBsAg levels.

## **Materials and Methods**

### **Patients and study design**

This was a randomized controlled trial. The sample size was determined using two samples t-test power calculation. The 1 log increase of serum HBV DNA level was defined as a significant difference between two samples. Effect size (Cohen's  $d=0.39$ ) was obtained using two parameters including difference and standard deviation. The power and a significant level were set as 0.8 and 0.05, respectively. The sample size was calculated out as 82 in each group. The ratio of exclusion was estimated as 20 %. Therefore, the sample size was set as 196 in this clinical trial. In our clinical experience, the majority of vitamin D deficiency patients can achieve adequate serum vitamin D levels after two months vitamin D supplement. Furthermore, the liver dysfunction due to hepatitis flare or cirrhosis could be a confounding factor for the low serum vitamin D levels in HBV-infected patients. According to the above consideration, our study was designed to enroll 196 non-cirrhotic, HBV-infected patients with normal or minimally elevated serum ALT levels. Thus, chronic HBV patients receiving regular 6-month surveillance and without current antiviral treatment in the outpatient clinic of Taipei Tzu Chi Hospital were invited to join the study from

August 2017 to September 2018. All had positive hepatitis B surface antigen (HBsAg) for more than 6 months. Those with positive anti-hepatitis C virus (HCV) antibody, alcoholic liver disease, other known causes of chronic hepatitis or hepatocellular carcinoma were excluded. Cirrhosis was diagnosed based on histological findings or ultrasonographic evidence of nodular liver surface with coarse echotexture and splenomegaly. After obtaining written informed consent, levels of serum vitamin D, HBV DNA and quantitative HBsAg (qHBsAg) levels were measured. Those who had HBsAg loss, undetectable HBV DNA and adequate vitamin D level ( $\geq 30$  ng/mL) were excluded. The eligible subjects were randomized into two groups by using the computer random distribution. Patients in the vitamin D group received vitamin D supplement (D3, 2000 IU/day; Metagenics, Aliso Viejo, CA 92656) for 2 months and untreated patients were used as the control group. Levels of vitamin D, HBV DNA and qHBsAg before and after 2 months of treatment or follow-up were compared between these two groups. The primary endpoint was to identify any difference between two groups. Patients lost to follow-up or poor drug compliance (vitamin D intake amount  $< 80\%$ ) were excluded for per-protocol analysis. Both intention-to-treat and per-protocol analyses were conducted.

### **Laboratory testing**

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using routine automated methods (Dimension Flex<sup>®</sup> reagent cartridge, USA; SIEMENS, Dimension, NY, USA). The upper limit of normal of ALT was 63 U/L in men and 59 U/L in women. The HBeAg and Anti-HCV were assayed using Abbott ARCHITECT, Wiesbaden, Germany. The Fibrosis 4 index (FIB-4) was calculated base on the formula:  $FIB-4 = Age \text{ (years)} \times AST \text{ (U/L)} / [PLT(10^9/L) \times ALT^{1/2}(U/L)]$ . Based on our previous study,  $FIB-4 \geq 0.87$  was defined as the presence of significant fibrosis ( $\geq F2$ ).<sup>21</sup>

### **Quantification of serum vitamin D level**

Serum vitamin D level was measured using LIAISON 25OH vitamin D Total assay (DiaSorin Inc, USA) based on an automated chemiluminescent immunoassay (CLIA) technology. Serum vitamin D concentration  $< 20$  ng/ml was defined as deficiency,  $< 30$  and  $\geq 20$  ng/ml as insufficiency, whereas concentrations  $\geq 30$  ng/ml were considered as sufficiency.

### **Quantification of HBV DNA and HBsAg levels**

Serum HBV DNA level was quantified using Roche COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Molecular Systems, Inc, USA). The lower limit of quantification was 20 IU/ml. Serum qHBsAg level was assayed using the Architect HBsAg QT (Abbott Laboratories) according to the manufacturer's instruction. The detection range is from 0.05 to 250 IU/ml. If the qHBsAg level was found to be higher than 250 IU/ml, the samples were 1:100 or 1:1000 diluted to obtain a reading within the range of the calibration curve.

### **Ethical considerations**

The study was performed in accordance with the principles of the 1975 Declaration of Helsinki and approved by the Ethical Committee of Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation. Each participant provided written informed consent at the time of enrollment. The register number of clinical trial is NCT03068767.



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