Effect of vitamin D supplementation in patients with chronic hepatitis C after direct-acting antiviral treatment: a randomized, double-blind, placebo-controlled trial

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Background. Replacement of vitamin D (VD) among patients with chronic hepatitis C (CHC) before viral eradication has demonstrated a protective effect on serum markers associated with hepatic fibrogenesis. We therefore hypothesized that VD may facilitate further fibrosis amelioration following curative treatment with direct-acting antivirals (DAA).

Methods. This study was a randomized, double-blind, placebo-controlled trial conducted between February 2018 and August 2018. Patients with CHC and VD deficiency were randomized in a 1:1 ratio to either receive ergicalciferol or placebo over 6 weeks. Biochemical analysis indicators, including 25-hydroxyvitamin D (25(OH)D), fibrogenic markers [(transforming growth factor beta 1 (TGF-β1) and tissue inhibitors of matrix metalloproteinases 1 (TIMP-1)], and fibrolytic markers [matrix metalloproteinase 9 (MMP-9) and amino terminal type III procollagen peptide (P3NP)], were assessed at baseline and at 6 weeks. Serum 25(OH)D was analyzed by a chemiluminescence immunoassay. Serum hepatic fibrogenesis markers were measured using a quantitative sandwich enzyme-linked immunosorbent assay.

Results. Seventy-five patients with CHC and VD deficiency were randomly assigned to VD (n=37) and placebo (n=38) groups. At the end of the study, the mean serum 25(OH)D level had risen to a normal level in the VD group, but was still deficient in the placebo group (41.8±9.1 vs. 18.1±4.6 ng/mL, p<0.001). Upon restoration of the VD level, there were no significant mean differences in the change from baseline for TGF-β1 (-0.6 ng/mL (95% confidence interval (95%CI) -2.8 – 1.7), p=0.63), TIMP-1 (-5.5 ng/mL (95%CI -26.4 – 15.3), p=0.60), MMP-9 (122.9 ng/mL (95%CI -69.0 – 314.8), p=0.21), and P3NP (-0.1 ng/mL (95%CI -2.4 – 2.2), p=0.92) between the VD and placebo groups.

Conclusion. Short-term VD supplementation after DAA treatment in patients with CHC does not improve serum fibrogenesis markers and may not expedite the residual liver fibrosis healing process. Future studies are warranted to evaluate the long-term effect of VD supplementation on hepatic fibrosis regression.

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26 **Abstract**

27 **Background.** Replacement of vitamin D (VD) among patients with chronic hepatitis C (CHC)
28 before viral eradication has demonstrated a protective effect on serum markers associated with
29 hepatic fibrogenesis. We therefore hypothesized that VD may facilitate further fibrosis
30 amelioration following curative treatment with direct-acting antivirals (DAA).
31 **Methods.** This study was a randomized, double-blind, placebo-controlled trial conducted
32 between February 2018 and August 2018. Patients with CHC and VD deficiency were
33 randomized in a 1:1 ratio to either receive ergicalciferol or placebo over 6 weeks. Biochemical
34 analysis indicators, including 25-hydroxyvitamin D (25(OH)D), fibrogenic markers
35 [(transforming growth factor beta 1 (TGF-β1) and tissue inhibitors of matrix metalloproteinases
36 1 (TIMP-1)], and fibrolytic markers [matrix metalloproteinase 9 (MMP-9) and amino terminal
37 type III procollagen peptide (P3NP)], were assessed at baseline and at 6 weeks. Serum 25(OH)D
38 was analyzed by a chemiluminescence immunoassay. Serum hepatic fibrogenesis markers were
39 measured using a quantitative sandwich enzyme-linked immunosorbent assay.

40   **Results.** Seventy-five patients with CHC and VD deficiency were randomly assigned to VD
41   (n=37) and placebo (n=38) groups. At the end of the study, the mean serum 25(OH)D level had
42   risen to a normal level in the VD group, but was still deficient in the placebo group (41.8±9.1 vs.
43   18.1±4.6 ng/mL, p<0.001). Upon restoration of the VD level, there were no significant mean
44   differences in the change from baseline for TGF-β1 (-0.6 ng/mL (95% confidence interval
45   (95%CI) -2.8 – 1.7), p=0.63), TIMP-1 (-5.5 ng/mL (95%CI -26.4 – 15.3), p=0.60), MMP-9
46   (122.9 ng/mL (95%CI -69.0 – 314.8), p=0.21), and P3NP (-0.1 ng/mL (95%CI -2.4 – 2.2),
47   p=0.92) between the VD and placebo groups.
48   **Conclusion.** Short-term VD supplementation after DAA treatment in patients with CHC does
49   not improve serum fibrogenesis markers and may not expedite the residual liver fibrosis healing
50   process. Future studies are warranted to evaluate the long-term effect of VD supplementation on
51   hepatic fibrosis regression.

52

Introduction

54   Hepatic fibrogenesis is a complex, dynamic cellular process regulated by a balance between
55   fibrogenic and fibrolytic activities. Liver fibrogenic cytokines, such as transforming growth factor
56   beta 1 (TGF-β1) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), initially activate the
57   quiescent hepatic stellate cells (HSCs) after liver injury. This process consequently leads to a
58   change in the fibrotic cascade and results in an excessive accumulation of extracellular matrix
59   (ECM) within the liver parenchyma (Lee et al. 2015). Subsequently, the cellular healing process
60   eventually occurs after the resolution of the injury. Matrix metalloproteinases (MMPs), which are
61   important fibrolytic enzymes, play a pivotal role in matrix remodeling during the recovery phase.
62   MMPs regulate collagen degradation, thereby facilitating liver fibrosis regression (Okazaki et al.
63   2000).

64

65   Hepatitis C virus (HCV) infection remains a major health-care problem worldwide, leading to high
66   morbidity and mortality from cirrhosis and hepatocellular carcinoma (HCC) (Stanaway et al.
67   2016). Notably, the advent of direct-acting antiviral (DAA) therapy for HCV has transformed
68   clinical practice and provided >90% cure rate among patients with chronic hepatitis C (CHC)
69   (European Association for the Study of the Liver. Electronic address & European Association for
70   the Study of the 2018). Amelioration of liver inflammation and improvement of hepatic synthetic
71   function have been demonstrated during the course of treatment and shortly after the completion
72   of DAA therapy (Charlton et al. 2015; Curry et al. 2015). Additionally, a remarkable reduction in
73   liver stiffness has also been shown after successful HCV eradication (Dolmazashvili et al. 2017;
74   Pan et al. 2018). However, a large number of patients with CHC still have substantial residual liver
75   fibrosis after the viral cure, thereby remaining as an important concern in current clinical practice
76   (Giannini et al. 2019; Singh et al. 2018). Recent studies have demonstrated that patients with CHC
77   who still have advanced liver fibrosis and cirrhosis after a sustained virological response (SVR)
78   remain at high risk of HCC (Su & Ioannou 2019). Thus, guidelines recommend continuing an HCC

79 surveillance program for such patients (European Association for the Study of the Liver. Electronic
80 address & European Association for the Study of the 2018; Panel 2018).
81
82 Vitamin D (VD) deficiency/insufficiency is a global problem affecting approximately 1 billion
83 people; its prevalence varies by age and ethnicity in different countries (Amrein et al. 2020). A
84 number of studies have reported a high prevalence of VD deficiency in patients with chronic liver
85 diseases, regardless of etiology (Arteh et al. 2010; Malham et al. 2011). Notably, >60% of patients
86 with CHC experience VD deficiency (Jin et al. 2018). Low serum VD levels could lead to negative
87 liver-related consequences and liver fibrosis progression (Dadabhai et al. 2017; Kubesch et al.
88 2018). Moreover, in a recent clinical study, since the liver is an intermediate organ of VD
89 metabolism, a high proportion of patients with CHC still have VD deficiency and insufficiency
90 after successful HCV eradication (Backstedt et al. 2017; Sriphoosanaphan et al. 2020).
91
92 Growing experimental and clinical evidence has revealed an anti-fibrogenic effect of VD (Baur et
93 al. 2012; Ding et al. 2013; Wahsh et al. 2016). Our previous study demonstrated a considerable
94 improvement in serum fibrogenesis markers after VD restoration in patients with CHC before
95 HCV treatment (Komolmit et al. 2017). This improvement could be due to the influence of VD on
96 HCV replication, inflammation reduction, or the direct effect of VD on hepatic fibrogenesis. In
97 this study, we aimed to further clarify the exact role of VD in hepatic fibrogenesis amelioration in
98 patients with CHC who underwent curative treatment with DAA.
99

Materials & Methods

Study population and study design

102 This randomized, double-blind, placebo-controlled study was conducted at King Chulalongkorn
103 Memorial Hospital, which is a tertiary referral center and academic teaching hospital, in Bangkok,
104 Thailand, from February 2018 to August 2018. All patients with CHC who achieved SVR within
105 1 year after DAA therapy in the hepatology clinic were assessed for eligibility. The inclusion
106 criteria were as follows: age between 18 and 80 years, VD insufficiency/deficiency (defined as
107 serum 25(OH)D level <30 ng/mL), and evidence of more than F2 liver fibrosis from either
108 Fibroscan® (transient elastogram ≥7.1 kPa (Castera et al. 2005)), ultrasound elastography (≥6.7
109 kPa by 2D shear-wave elastography (Bende et al. 2017)), or magnetic resonance elastography
110 (≥3.2 kPa by two-dimensional gradient recall echo (Ichikawa et al. 2012)). Patients with a recent
111 history of VD supplementation, evidence of chronic hepatitis B or human immunodeficiency virus
112 infection, decompensated liver cirrhosis, autoimmune diseases, hepatocellular carcinoma, active
113 infections from other pathogens, a history of steroid or immunosuppressive agent use, or a history
114 of interferon treatment within 12 months, and those who were pregnant or lactating, were excluded.
115
116 Data were collected as previously described in Komolmit et al. (2017). Specifically, randomization
117 was performed with a computer-generated allocation sequence. The eligible patients were
118 randomly assigned to receive VD or placebo (1:1 allocation using a random block size of 4). The

119 process was performed by a research assistant who was not involved in the study. Randomization
120 codes were secured in sealed envelopes until all data entry was complete.
121
122 **Intervention**
123 Vitamin D2 (VD2, ergocalciferol) and the placebo were prepared in identical capsules of the same
124 weight by the Department of Pharmacy at King Chulalongkorn Memorial Hospital. All
125 investigators and patients were blinded to the type of medication used until the end of the study.
126 Each capsule of VD2 contained 20,000 international units of ergocalciferol. The protocol for VD
127 supplementation was based on the 25(OH)D level at baseline (Table 1), which has been previously
128 demonstrated to effectively increase VD levels in patients with CHC within a 6-week period
129 (Komolmit et al. 2017). All patients initially received ergocalciferol or placebo on the day of
130 randomization.
131
132 Baseline clinical characteristics, HCV genotypes, liver enzymes, and fibrosis-4 (FIB-4) and
133 aspartate aminotransferase to platelet ratio index (APRI) scores were assessed. Blood samples
134 were collected at two time points (baseline and at the end of the 6-week follow-up) and were kept
135 at −70°C until analysis. All blood samples were simultaneously analyzed for TGF-β1, TIMP-1,
136 MMP-9, and amino terminal type III procollagen peptide (P3NP) levels at the end of the study.
137 Pill counts and patient interviews were used to assess adherence to the prescribed medications. All
138 participants were asked to refrain from outside-of-trial VD or multivitamin supplements and to
139 maintain their normal activity. At the end of the study, patients who remained in a VD-insufficient
140 state received VD supplementation as a standard of care.
141
142 **Outcomes and assessments**
143 The primary outcome was the effect of VD supplementation on serum markers associated with
144 hepatic fibrogenesis, including TGF-β1, TIMP- 1, MMP-9, and P3NP, relative to the effect in the
145 placebo group at the 6-week follow-up.
146
147 **Laboratory assessment**
148 **Measurement of 25(OH)D level**
149 Serum 25(OH)D levels, a functional indicator of VD status, were measured using the Liaison
150 25(OH) vitamin D total assay (DiaSorin, Saluggia, Italy); the measurement was performed on the
151 LIAISON® chemiluminescence analyzer following the manufacturer's instructions. The final
152 level is reported in ng/mL. According to the Endocrine Society Practice Guidelines (Holick et al.
153 2011), VD insufficiency/deficiency was defined as a 25(OH)D level <30 ng/mL. Specifically,
154 serum 25(OH)D levels <20 ng/mL and between 20–29 ng/mL were defined as VD deficiency and
155 VD insufficiency, respectively.
156
157 **Measurement of TGF-β1 level**

158   The serum levels of human activated TGF-β1 were measured with a quantitative sandwich
159   enzyme-linked immunosorbent assay (ELISA) technique following the manufacturer's
160   instructions (Quantikine® ELISA, R&D Systems, Minneapolis, MN, USA). Before the assay, the
161   latent TGF-β1 contained in the patients' serum was activated to the immunoreactive form using
162   acid activation and neutralization. The results were calculated by reference to the standard curve.
163

164   **Measurement of TIMP-1 level**
165   The serum levels of human TIMP-1 were measured using the quantitative sandwich ELISA
166   technique according to the manufacturer's instructions (Quantikine® ELISA). The results were
167   calculated by reference to the standard curve.
168

169   **Measurement of MMP-9 level**
170   The serum levels of human MMP-9 protein were measured by a quantitative sandwich ELISA
171   according to the manufacturer's instructions (Quantikine® ELISA). The results were calculated
172   by reference to the standard curve.
173

174   **Measurement of P3NP level**
175   The serum levels of P3NP were measured via a quantitative sandwich ELISA technique according
176   to the manufacturer's instructions (Cloud-Clone Corp., TX, USA). The results were calculated by
177   reference to the standard curve.
178

179   **Sample size calculation**
180   Sample size calculations were based on data from our previous study on patients with CHC
181   (Komolmit et al. 2017). Of the cytokines measured in both the present study and our previous
182   study, we used the data of TGF-β1 for the calculation, since it resulted in the largest sample size.
183   The mean difference in the change in TGF-β1 level from baseline to week 6 in the VD
184   supplementation arm versus the placebo arm as a control was 81 pg/mL, with a pooled standard
185   deviation of 110. Thus, enrolling 30 patients per group would give 80% power to detect a
186   difference in TGF-β1 change from baseline between treatment groups of this size or more, at a 2-
187   sided significance level of 5%. We inflated the sample size to 37 per randomized arm to account
188   for possible dropouts.
189

190   **Statistical analysis**
191   Baseline characteristics, including clinical and laboratory data, are presented as percentage or
192   mean ± standard deviation (SD). In the initial analyses, the baseline characteristics of the patients
193   were compared using t-tests or chi-square tests. Categorical variables were analyzed using the chi-
194   square test or Fisher's exact test, as appropriate. For the primary study outcome of fibrogenesis
195   marker changes, we used the independent *t*-test to evaluate mean differences and 95% confidence
196   interval (95%CI) in the change from baseline to week 6 between the VD and the placebo groups.
197   As a sensitivity analysis, to ensure that outliers did not exert an undue influence on the obtained

198 between-arm estimates, we summarized the data as the median and 25th and 75th percentile levels,
199 and used quantile regression to compare the median serum level changes between randomized
200 arms. Statistical analyses were performed with Stata 16.1 (Statacorp, College Station, TX, USA).
201
202 **Ethical approval**
203 This study was reviewed and approved by the Ethics Committee and Institutional Review Board
204 (IRB) at Chulalongkorn University, Bangkok, Thailand, and was performed in accordance with
205 the Declaration of Helsinki (1989) of the World Medical Association (IRB number: 707/60).
206 Moreover, this trial was registered with the Thai Clinical Trials Registry (TCTR) based on the
207 World Health Organization criteria on 22 November 2017 (TCTR20171206003). Prior to
208 participation, all patients enrolled in this study provided written informed consent for participation,
209 as well as for publication.
210
211 **Results**
212 **Patient characteristics**
213 Between February 2018 and August 2018, 80 patients with CHC who achieved SVR after DAA
214 treatment were screened for eligibility. Five patients were excluded from the study (Fig 1). A total
215 of 75 patients were finally included and randomly assigned to the VD group (n=37) or the placebo
216 group (n=38). Mean age was 60.4±7.8 years, patients were mostly female (n= 45, 60%), and a
217 majority of the participants had cirrhosis (n=45, 60%). As shown in Table 2, baseline
218 characteristics between the groups were well balanced. No significant difference in any of the
219 biochemical parameters and degree of liver fibrosis, as measured by FIB-4 and APRI scores, was
220 found between the groups.
221
222 **Changes in VD levels**
223 Mean baseline serum 25(OH)D level was 17.2±4.8 ng/mL and 16.6±4.1 ng/mL in the VD and
224 placebo groups, respectively. At the 6-week follow-up, the mean 25(OH)D level in the VD group
225 increased to a normal level (41.8±9.1 ng/mL), whereas a slight increase was observed in the
226 placebo group (18.1±4.6 ng/mL) (Fig 2). The mean difference in 25(OH)D change from baseline
227 to week 6 reflected a significantly greater change in the VD group than in the placebo group (23.1
228 ng/mL (95%CI 19.7 – 26.4), p<0.001).
229
230 Of note, three patients (8.1%) in the VD group still had a serum 25(OH)D level <30 ng/mL at 6
231 weeks after VD replacement; the 25(OH)D levels in two patients changed from an initial deficient
232 state to an insufficient state after VD supplementation (absolute VD increase of 18.3 and 18.4
233 ng/mL, respectively), while the remaining patient had a slightly increased VD level (from 21.8
234 ng/mL at baseline to 23.5 ng/mL) after VD replacement. All participants in the placebo group still
235 had VD insufficiency/deficiency. All unused pills were returned at the last follow-up, and no
236 patient reported missing any medications.
237

238 **Changes in serum hepatic fibrogenesis markers**
239 Mean and median differences in serum marker changes from baseline to 6 weeks are shown in
240 Tables 3 and 4, respectively, and in Figure 3. Serum TGF-β1 levels slightly increased from
241 16.3±6.6 ng/mL at baseline to 16.8±7.6 ng/mL at 6 weeks in the VD group, and increased from
242 14.3±8.3 to 15.4±6.8 ng/mL in the placebo group. However, there was no significant difference in
243 the mean change from baseline between the VD and placebo groups (-0.6 ng/mL (95%CI -2.8 –
244 1.7), p=0.63).

245

246 Similarly, mean serum TIMP-1 levels in the VD group slightly increased from 236.9±50.0 ng/mL
247 at baseline to 244.0±66.0 ng/mL at 6 weeks. In the placebo group, mean serum TIMP-1 levels
248 slightly increased from 231.9±50.3 ng/mL at baseline to 244.5±63.6 ng/mL at 6 weeks. There was
249 no significant difference in the mean TIMP-1 change from baseline to 6 weeks between the VD
250 and placebo groups (-5.5 ng/mL (95%CI -26.4 – 15.3), p=0.60).

251

252 Regarding fibrolytic markers, mean MMP-9 levels showed an increasing trend in the VD group
253 (576.2±296.2 ng/mL at baseline and 684.3±423.5 ng/mL at 6 weeks); median levels showed a
254 consistent pattern. In contrast, mean MMP-9 levels in the placebo group were approximately the
255 same at baseline and week 6, but the median levels increased. There was no significant difference
256 in the mean change from baseline at week 6 between the VD and placebo groups (122.9 ng/mL
257 (95%CI -69.0 – 314.8), p=0.21).

258

259 Mean P3NP levels slightly decreased from the baseline in both groups. There was no significant
260 difference in the mean P3NP change score from baseline to week 6 between the VD and placebo
261 groups (-0.1 ng/mL (95%CI -2.4 – 2.2), p=0.92).

262

263 **Sensitivity analysis**
264 We conducted quantile regression, using the median as another measure of the central tendency,
265 to assess whether extreme outliers in the dataset had a strong influence on the observed
266 associations. In both VD and placebo groups, the median, 25th and 75th percentile values of each
267 marker were very similar at baseline and week 6 visits (Fig. 3, Table 4). The differences in the
268 mean and median changes in marker concentrations from baseline to week 6 between randomized
269 arms were of similar magnitude and direction, with the exception of MMP-9. For this latter marker,
270 the mean change was greater, but the median change was smaller, in the VD group than in the
271 placebo group. However, neither result was statistically significant, and the 95%CI around both
272 the mean and median differences were wide.

273

274 **Subgroup analysis**
275 A subgroup analysis was performed according to the severity of VD deficiency/insufficiency,
276 which showed no significant changes in serum hepatic fibrogenesis markers in both groups.

277 Additional subgroup analyses based on sex, cirrhosis, liver fibrosis severity, and a 25(OH)D level
278 <10 ng/ml at baseline also revealed similar results (Table S1 and S2).
279

Discussion

281 In this randomized, double-blind, placebo-controlled study, 6-week VD supplementation in
282 patients with CHC after HCV eradication by DAA did not help attenuate liver fibrosis as assessed
283 by fibrogenesis markers. Additionally, the effect of VD supplementation did not vary according to
284 baseline serum 25(OH)D levels and liver fibrosis severity.
285

286 Activation of HSCs to myofibroblast-like cells by TGF-β1 results in fibrillary component
287 accumulation, which leads to progressive liver fibrosis (Lee et al. 2015). TGF-β1 also regulates
288 TIMP-1 production, which subsequently inhibits the breakdown of excessive ECM (Flisiak et al.
289 2005). In contrast, a shift toward fibrolytic activity results in the reversal of liver fibrosis. MMPs
290 function as the main enzymes responsible for fibrillary matrix cleavage (Wynn 2007). P3NP is a
291 breakdown peptide produced during the healing process, and is widely used as a marker for hepatic
292 fibrolysis. A decline in P3NP levels correlates with an increase in cellular fibrosis degradation and
293 indicates liver fibrosis regression (Nielsen et al. 2015). Based on their involvement in hepatic
294 fibrogenesis, these cellular cytokines and enzymes have been used as markers for an indirect
295 assessment of hepatic fibrogenesis (Komolmit et al. 2017; Liu et al. 2012)
296

297 Apart from its classical action on calcium metabolism, VD is involved in various cellular
298 regulatory processes. Immunomodulatory, anti-inflammatory, and anti-fibrotic properties of VD
299 have been reported in numerous studies. VD plays a crucial role in the functioning of the innate
300 and adaptive immune systems (Aranow 2011; Holick 2007). Further, VD has also been
301 demonstrated as an innate antiviral agent; it exerts an inhibitory effect on HCV replication in
302 human cells (Gal-Tanamy et al. 2011). Regarding the association of VD and hepatic fibrogenesis,
303 VD receptor (VDR) mRNA in HSCs is upregulated in response to TGF-β1 (Beilfuss et al. 2015).
304 A study by Ding et al. showed spontaneous liver cirrhosis in *VDR* knockout mice, which was
305 mediated by the TGF-β/VDR/SMAD signaling circuit (Ding et al. 2013). In addition, 1,25(OH)D
306 stimulated VDR expression in primary rat HSCs and suppressed TIMP-1 mRNA expression,
307 resulting in an improvement in fibrotic score in thioacetamide-induced liver fibrosis (Abramovitch
308 et al. 2011). Furthermore, a synthetic VD analogue, calcipotriol, has been found to prevent liver
309 cirrhosis in a mouse model (Wahsh et al. 2016). In a human study, Beilfuss et al. demonstrated the
310 role of VD in liver fibrogenesis via the crosstalk between VD and the TGF-β1 signaling pathway.
311 With VD supplementation, a reduced fibrogenic response in human HSCs was observed (Beilfuss
312 et al. 2015). Based on the aforementioned evidence, at least three main properties of VD that could
313 influence the fibrinolytic state can be postulated: anti-viral (i.e., suppression of viral replication),
314 anti-inflammatory, and anti-fibrotic properties.
315

316 In our previous study, restoration of VD levels in patients with CHC resulted in dynamic shifts of
317 serum fibrosis markers toward anti-fibrotic activity. The levels of pro-fibrotic cytokines (i.e. TGF-
318 β1 and TIMP-1) significantly decreased in the VD supplementation group, whereas the levels of
319 anti-fibrotic enzymes (i.e. MMP-2 and MMP-9) were significantly higher in the VD
320 supplementation group than in the placebo group (Komolmit et al. 2017). This evidence highlights
321 and supports the protective role of VD in the hepatic fibrogenesis process. In our current study, we
322 aimed to explore the effect of VD replacement after curative HCV therapy in order to ascertain
323 whether VD mainly facilitates fibrolytic processes by halting fibrogenesis, not by its effect on
324 HCV replication or inflammation. Based on our results, VD restoration after HCV eradication did
325 not cause significant changes in serum markers associated with hepatic fibrogenesis. Hence, one
326 could assume that VD, at the clinical level, might not help facilitate hepatic fibrolysis. In addition,
327 the effect of VD on the improvement in fibrogenesis markers, as shown in our previous study,
328 might not be directly achieved via the intrinsic fibrogenesis cascades, as observed in several
329 experimental results. Thus, apart from HCV eradication and the resolution of liver inflammation,
330 VD restoration after DAA treatment may not provide any further benefit in the reversal of hepatic
331 fibrogenesis. This seems to suggest that VD might regulate and influence hepatic fibrogenesis
332 through its immunomodulatory rather than its anti-fibrotic property. While VD might have a role
333 in hepatic fibrosis regulation, its contribution is not strong enough to affect the associated serum
334 markers. Investigation on changes in local tissue levels to elucidate this observation may be
335 warranted.

336

337 The baseline TGF-β1 levels in the current cohort could be another possible explanation for the
338 major difference in results between our previous and current studies. Unlike our previous study,
339 the baseline TGF-β1 levels were similar to those observed in healthy volunteers (Flisiak et al.
340 2005; Grainger et al. 2000), and were consistent with the levels in successfully treated patients
341 with CHC in studies conducted in the interferon era (Flisiak et al. 2005; Kotsiri et al. 2016). Given
342 the normalized baseline serum TGF-β1 levels, a large number of participants would be needed to
343 detect differences in the outcome of interest. It is more likely that, at this stage in clinical trials, an
344 investigation into the hepatic tissue expression levels of each marker would be appropriate to
345 further test our hypothesis. However, in the era of DAA therapy for CHC, a paired liver biopsy
346 has ethical implications.

347

348 In contrast to TGF-β1 levels, the MMP-9 and P3NP levels at baseline were higher than those in
349 healthy population (Latronico et al. 2016; Teare et al. 1993). A closer analysis of the hepatic
350 fibrolytic processes revealed a trend towards fibrosis degradation as evidenced by the changes in
351 MMP-9 and P3NP levels; however, the changes did not reach statistical significance.

352

353 Apart from VD levels, genetic variation in the *VDR* gene might be one of the factors with an
354 influence on the study findings. Since *VDR* polymorphism has been shown to be associated with
355 liver fibrosis and potentially determines the VD response (Baur et al. 2012; Estrabaud et al. 2012),

356 the negative results of the current study might be partly due to *VDR* genetic variation. However,
357 this assumption cannot be confirmed in the current clinical setting.
358
359 To the best of our knowledge, this is the first study to determine the effect of VD on liver fibrosis
360 in patients with CHC after DAA treatment. We presented some additional evidence on the
361 influence of VD on hepatic fibrogenesis. Nevertheless, this study has some limitations. First,
362 several factors, such as diet, sunlight exposure, and supplementary products, could affect the
363 measured serum VD levels. To minimize the possible influence of these factors and other potential
364 biases, we employed a double-blind, placebo-controlled study design. Moreover, the participants
365 were advised to maintain their usual activities of daily living and avoid any supplements during
366 the study period. Second, histological evidence in the liver parenchyma would ideally be a gold
367 standard to evaluate liver fibrosis. However, paired liver biopsy is an invasive procedure, and thus,
368 is not free of risk. Owing to ethical and practical considerations, we decided to employ a non-
369 invasive approach using surrogate serum markers to evaluate fibrogenesis. Lastly, we selected a
370 6-week time point because, in our previous study, VD levels were normalized in most of the
371 patients by VD supplementation within this time period. As hepatic fibrogenesis is highly dynamic,
372 and the half-lives of serum fibrogenesis cytokines and enzymes are relatively short, with spans of
373 hours and days, we believe that a local immunological change would be initially demonstrated
374 within this time period. However, since the reversal of hepatic fibrogenesis could take months or
375 years to show a clinical benefit, a short follow-up period may be inadequate for detecting any
376 significant difference. Hence, future long-term studies are needed to further our understanding.
377

378 ## Conclusions
379 Short-term VD replacement after HCV eradication by DAA did not improve serum hepatic
380 fibrogenesis marker levels and, thus, might not clinically facilitate the amelioration of residual
381 liver fibrosis. Nevertheless, further investigation to determine whether a higher dose (VD
382 derivative) for a longer period could potentially help ameliorate liver fibrosis is warranted.
383

384 ## Acknowledgements
385 We would like to express our gratitude to the staff of the Division of Gastroenterology, Department
386 of Medicine, Center of Excellence in Liver Diseases, King Chulalongkorn Memorial Hospital, and
387 Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University for
388 their technical assistance and clinical support.
389

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542

Protocol for VD supplementation

1   **Table 1.** Protocol for VD supplementation

Diagnosis	25(OH)D level (ng/mL)	Replacement (IU/week)	Ergocalciferol 20,000 IU/tab	Duration
Insufficiency	20–30	60,000	2 tabs Monday and 1 tab Friday	6 weeks
Deficiency	10–20	80,000	2 tabs Monday and 2 tabs Friday	6 weeks
Severe deficiency	<10	100,000	3 tabs Monday and 2 tabs Friday	6 weeks

4   * VD: vitamin D; 25(OH)D: 25-hydroxyvitamin D; IU: international unit

Baseline characteristics

1 **Table 2.** Baseline characteristics

	VD group (n=37)	Placebo (n=38)
Age (years)	60.9±7.8	59.4±8.0
Sex, male	16 (43.2%)	14 (36.8%)
Body mass index (kg/m^2)	25.3±4.3	24.3±4.1
HCV viral load before treatment (log IU/mL)	5.9±0.9	5.5±1.4
Genotype (n)		
1	19	18
3	17	17
6	1	3
Cirrhosis	21 (56.8%)	24 (63.2%)
AST (U/L)	29.6±16.0	28.9±10.9
ALT (U/L)	23.5±11.0	23.7±10.0
DAA regimen		
SOF/DAC	11	4
SOF/LED	1	3
SOF/VEL	0	0
SOF/DAC/RBV	23	23
SOF/LED/RBV	1	7
SOF/VEL/RBV	1	1
Fibrosis 4 score (FIB-4)	2.8±2.1	3.4±2.8
AST to platelet ratio index (APRI)	0.6±0.5	0.7±0.6
25(OH)D level (ng/mL)	17.2±4.8	16.6±4.1
TGF-β1 level (ng/mL)	16.3±6.6	14.3±8.3
TIMP-1 level (ng/mL)	236.9±50.0	231.9±50.3
MMP-9 level (ng/mL)	576.2±296.2	570.1±477.7
P3NP level (ng/mL)	28.6±5.7	28.0±4.3

4 *VD: vitamin D; HCV: Hepatitis C virus; IU: international unit; AST: aspartate aminotransferase; ALT:
5 alanine aminotransferase; DAA: direct-acting antiviral; SOF: sofosbuvir; DAC: daclatasvir; LED:
6 ledipasvir; VEL: velpatasvir; RBV: ribavirin; 25(OH)D: 25-hydroxyvitamin D; TGF-β1: transforming
7 growth factor beta 1; TIMP-1: tissue inhibitor of matrix metalloproteinase-1; MMP-9: matrix
8 metalloproteinase 9; P3NP: amino terminal type III procollagen peptide

Serum biochemical markers levels at baseline and 6 weeks of follow-up, and the mean difference (95%CI) in serum marker change from baseline to 6 weeks in the VD versus the placebo arm as a reference

1 **Table 3.** Serum biochemical markers levels at baseline and 6 weeks of follow-up, and the mean difference
2 (95%CI) in serum marker change from baseline to 6 weeks in the vitamin D (VD) versus the placebo arm
3 as a reference.

Serum marker	Week	VD (n=37)	Placebo (n=38)	Mean difference (95%CI) in change from baseline to 6 weeks in VD vs placebo arm	p value
25(OH)D level (ng/mL)	0	17.2±4.8	16.6±4.1		
	6	41.8±9.1	18.1±4.6	23.1 (19.7 – 26.4)	<0.001
AST (U/L)	0	29.6±16.0	28.9±10.9		
	6	29.9±15.6	30.0±12.5	-0.7 (-4.2 – 2.8)	0.69
ALT (U/L)	0	23.5±11.0	23.7±10.0		
	6	23.8±11.1	27.1±14.2	-3.0 (-7.5 – 15)	0.20
TGF-β1 level (ng/mL)	0	16.3±6.6	14.3±8.3		
	6	16.8±7.6	15.4±6.8	-0.6 (-2.8 – 1.7)	0.63
TIMP-1 level (ng/mL)	0	236.9±50.0	231.9±50.3		
	6	244.0±66.0	244.5±63.6	-5.5 (-26.4 – 15.3)	0.60
MMP-9 level (ng/mL)	0	576.2±296.2	570.1±477.7		
	6	684.3±423.5	555.2±290.5	122.9 (-69.0 – 314.8)	0.21
P3NP level (ng/mL)	0	28.6±5.7	28.0±4.3		
	6	27.6±6.4	27.2±4.3	-0.1 (-2.4 – 2.2)	0.92

6 *VD: vitamin D; 25(OH)D: 25-hydroxyvitamin D; AST: aspartate aminotransferase; ALT: alanine
7 aminotransferase; TGF-β1: transforming growth factor beta 1; TIMP-1: tissue inhibitor of matrix
8 metalloproteinase-1; MMP-9: matrix metalloproteinase 9; P3NP: amino terminal type III procollagen
9 peptide

Sensitivity analysis

The median (25th-75^{th}percentile) serum biochemical marker levels at baseline and 6 weeks, and the median change (95%CI) in serum biochemical marker levels from baseline to 6 weeks of follow-up, in the VD versus the placebo arm as a reference, from median quantile regression

1 **Table 4.** Sensitivity analysis showing the median (25th-75th percentile) serum biochemical marker levels at
2 baseline and 6 weeks, and the median change (95%CI) in serum biochemical marker levels from baseline
3 to 6 weeks of follow-up, in the Vitamin D (VD) versus the placebo arm as a reference, from median quantile
4 regression.

Serum Marker	Week	VD Median (IQR)	Placebo Median (IQR)	Median difference (95%CI) in change from baseline to 6 weeks in VD vs placebo arm	p value
25(OH)D level (ng/mL)	0	19.2 (19.2 –21.3)	16.9 (14 – 19.1)		
	6	39.5 (36.7 – 46.5)	17.6 (15.1 – 21.1)	24.2 (20.8 - 27.6)	<0.001
TGF-β1 level (ng/mL)	0	14.8 (11.5 – 21.1)	15.0 (9.2 – 17.8)		
	6	15.8 (10.1 – 22.5)	15.6 (8.8 – 20.3)	-1.5 (-3.2 - 0.22)	0.09
TIMP-1 level (ng/mL)	0	232.0 (200.7 – 275.1)	226.4 (199.9 – 261.1)		
	6	229.0 (197.8 – 275.5)	229.2 (189.0 – 276.0)	-13.2 (-33.2 - 6.7)	0.19
MMP-9 level (ng/mL)	0	584.8 (345.2 – 802.1)	449.4 (339.0 – 637.5)		
	6	615.1 (329.5 – 890.8)	486.0 (363.7 -721.7)	-9.2 (-146 - 127.7)	0.89
P3NP level (ng/mL)	0	27.0 (26.1 – 31.0)	28.1 (25.6 – 31.0)		
	6	25.8 (24.1– 28.8)	26.6 (23.8 – 29.7)	-0.1 (-2.8 - 2.5)	0.92

6
7 * VD: vitamin D; 25(OH)D: 25-hydroxyvitamin D; TGF-β1: transforming growth factor beta 1; TIMP-1:
8 tissue inhibitor of matrix metalloproteinase-1; MMP-9: matrix metalloproteinase 9; P3NP: amino terminal
9 type III procollagen peptide

Figure 1

Screening, randomization, and follow-up
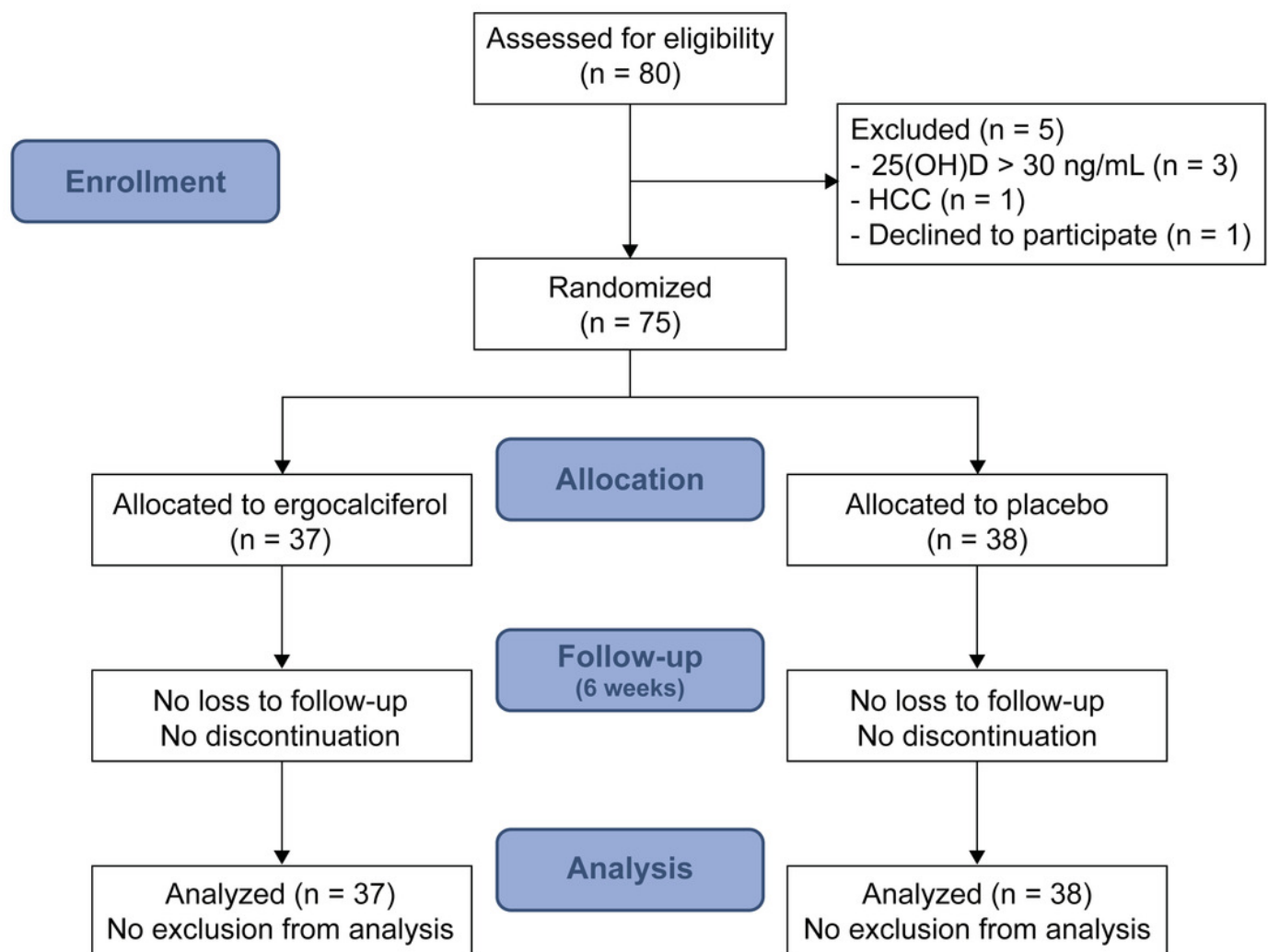
Figure 2

Box plot of serum 25(OH)D at baseline and after VD or placebo supplementation for 6 weeks
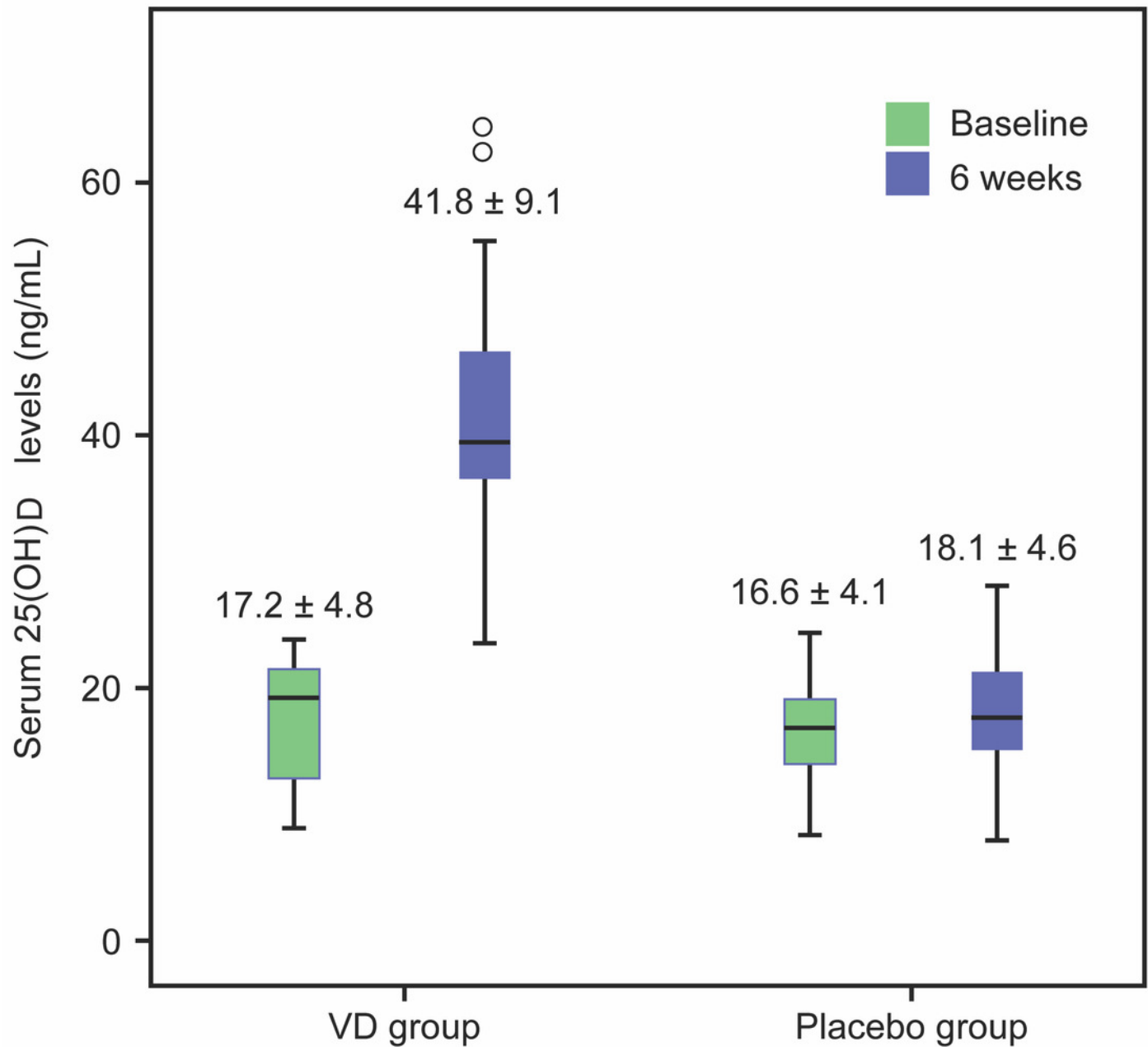
Figure 3

Line plot showing the individual serum marker levels for each patient connected with the levels at 6 weeks by randomized treatment arm. The overall median and mean levels at weeks 0 and 6 are connected by a dashed and solid line, respectively.

(A) TGF-β1. (B) TIMP-1. (C) MMP-9. (D) P3NP.