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CLINICAL TRIAL STUDY

Vitamin D and Calcium Supplement Attenuate Bone Loss among HIV-Infected Patients Receiving Tenofovir Disoproxil Fumarate/Emtricitabine/Efavirenz: An Open-Label, Randomized Controlled Trial

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Abstract: ***Background*:** Antiretroviral therapy (ART), especially with tenofovir disoproxil fumarate (TDF), has been associated with accelerated bone turnover and leads to significant bone loss.

***Objective*:** We aimed to determine the effect of vitamin D_2 and calcium on bone mineral density (BMD) in HIV-infected patients receiving TDF/emtricitabine (FTC)/efavirenz (EFV).

***Methods*:** A prospective, open-label, randomized controlled study was conducted. Eligible patients were ART naïve HIV individuals who initiated TDF/FTC/EFV. The study group received supplementation with vitamin D_2 and calcium carbonate, whereas the control group was administered only ART. The primary outcome was the percentage change in total hip BMD at week 24 compared with baseline.

***Results*:** A total of 18 patients were randomized (9 in each group). The mean (standard deviation; SD) total hip BMD significantly decreased from baseline in both groups, from 0.96 (0.14) g/cm^2 to 0.93 (0.13) g/cm^2 in the study group (p = 0.006) and from 0.87 (0.11) g/cm^2 to 0.84 (0.11) g/cm^2 in the control group (p = 0.004). The mean (SD) lumbar spine BMD significantly decreased from baseline in both groups, from 1.00 (0.13) g/cm^2 to 0.97 (0.13) g/cm^2 (p = 0.004) in the study group and from 0.90 (0.09) g/cm^3 to 0.86 (0.08) g/cm^2 in the control group (p = 0.006). At week 24, the mean (SD) lumbar spine BMD was significantly greater in the study group than in the control group (p = 0.042). However, there were no significant differences in the percentage change of total hip, lumbar spine, and femoral neck BMD between both groups. No adverse events were reported. In conclusion, as early as 24 weeks after TDF initiation, a significant decline in BMD was detected.

***Conclusion*:** Vitamin D_2 and calcium supplements should be considered for HIV-infected patients receiving TDF/FTC/EFV in a resource-limited setting where there are limited ART options (Clinicaltrials.gov NCT0287643).

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1. INTRODUCTION

Early initiation of antiretroviral therapy (ART) has improved substantially and transformed HIV infection into a manageable chronic disease [1-3]. Although the incidence of AIDS-related mortality has recently decreased [4-6], increased incidences of other comorbid conditions, including cardiovascular disease, metabolic complications, decreased bone mass, osteoporosis, and fragility fractures, have been observed [7-11]. A meta-analytical review of published cross-sectional studies from 2001-2005 reveals that the prevalence of osteoporosis is 15% in HIV-infected individuals [12, 13], and they have a greater risk of fractures than the general population [14-16]. Many recent studies reported that HIV/HCV-coinfection has significantly increased osteoporosis risk compared to HIV or HCV infection alone [17-20]. The risks of fracture in any sites and fragility fracture were also increased in HIV-infected patients [21]. Bone loss in HIV-infected individuals could be attributed to multiple causes, for example, direct effects of HIV on osteoclasts and

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osteoblasts as well as generalized inflammation, which promotes osteoblast apoptosis and reduces the functionality of these cells [22]. Overall, this mechanism shifts the bone-remodeling pathway toward bone resorption [22, 23]. Other factors contributing to bone loss including lifestyle, behavior, low body mass, comorbid conditions, and ART [24-27].

Several studies have suggested that initiation of ART is associated with a 2% to 6% reduction in bone mineral density (BMD) during the first 2 years of treatment, regardless of ART regimens and stabilization after that [5, 28-32]. This magnitude of bone loss was similar to that of postmenopausal women during the first year of the postmenopausal period [33]. A study comparing bone loss in HIV-infected individuals receiving different ART classes such as nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) found that NRTIs were associated with significantly greater BMD loss in total hip and spine than other classes of antiretroviral drugs [30]. Within the class of NRTIs, tenofovir disoproxil fumarate (TDF) has been associated with significant bone loss compared with other NRTIs in both HIV-infected and uninfected individuals [22, 28, 30, 34]. TDF-induced proximal renal tubular dysfunction triggers phosphaturia with accompanying hypophosphatemia [35], resulting in bone resorption in an attempt to liberate matrix phosphorus [24, 36]. Within the class of NNRTIs, an association with low vitamin D status has been reported for efavirenz (EFV) but not nevirapine or rilpivirine [10, 37-40]. Vitamin D plays central roles in various processes, including calcium homeostasis, regulation of bone turnover, immune function, cell proliferation, and cell differentiation [23]. For the prevention and treatment of osteoporosis, providing adequate daily calcium and vitamin D is a safe and inexpensive way to help reduce fracture risk [41]. Bone health is an indicator of optimal 25-hydroxyvitamin D [25(OH)D] levels, which vary from 20 to 30 ng/mL [41]. However, most guidelines for the prevention and treatment of osteoporosis recommend keeping 25(OH)D levels of at least 30 ng/mL to maintain BMD, reduce the risk of falling, and prevent osteoporotic fractures [41]. For PIs, some small studies have demonstrated an impact on BMD [24, 29, 42]. Meanwhile, integrase inhibitors seem to preserve bone health compared to other classes of ART [43, 44].

According to the current Thai guidelines for ART in HIV-infected adults, TDF/emtricitabine (FTC) or lamivudine (3TC)/EFV is the first-line regimen for ART naïve HIV individuals [45, 46]. The guidelines also recommend avoiding TDF and cautious use of EFV in patients with metabolic bone conditions [45, 46]. Abacavir (ABC) or tenofovir alafenamide (TAF) is recommended for individuals with a high risk of fracture and documented osteoporosis [47], since these drugs lead to less bone loss than other NRTIs do. However, TAF cannot be used in patients with tuberculosis who are treated with rifampicin [48], which is the most common opportunistic infection in many countries, including Thailand. Moreover, this drug is not available in many countries. In addition, the use of ABC is hampered by the need to test for HLA-B*5701 alleles before commencing the drug [49]. This test is costly and is performed only in certain centers in Thailand, including other resource-limited settings. Furthermore, according to the Department of Health and Human Services guidelines [49], avoidance of ABC in patients with high cardiac risk should be considered because some studies have revealed an association between ABC and myocardial infarction [50-52].

There is evidence that high dose vitamin D_3 (cholecalciferol) and calcium supplements can preserve the loss of hip BMD in ART naïve HIV-infected individuals who initiated TDF/FTC/EFV [33]. Nonetheless, the application of this study in the Thai population was limited by the fact that it was conducted in the United States and Puerto Rico, where are different in dietary sources of calcium, ethnicities and sunlight exposure. Furthermore, the use of vitamin D_3 supplement is not available in Thailand and some other countries. We hypothesized that vitamin D_2 (ergocalciferol), another form of vitamin D supplement, and calcium might attenuate bone loss in Thai HIV-infected patients who are currently taking TDF/FTC/EFV. The primary endpoint was the percentage change of total hip BMD at week 24 from baseline. The secondary endpoints were percentage change of spine BMD, femoral neck BMD, change in plasma total 25(OH)D, parathyroid hormone (PTH) levels, bone markers including procollagen type 1 amino-terminal propeptide (P1NP), and *C*-terminal cross-linking telopeptide of type 1 collagen (CTX) at week 24 from baseline.

2. MATERIALS AND METHODS

2.1. Study Design and Study Population

We conducted an open-label, randomized, single-center, controlled trial at Ramathibodi Hospital (a 1,200-bed university hospital in Bangkok, Thailand). We initiated enrollment of patients between June 2016 and December 2016, and they were followed up until June 2017. Ethical approvals of this study were obtained from committee on human rights related to research involving human subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (approval number: ID 01-59-11). All patients provided written informed consent prior to participation in the study.

The study compared the use of supplementation of vitamin D_2 and calcium with ART or the use of ART alone in HIV-infected individuals. Inclusion criteria included (1) age 18-50 years, (2) HIV-infected patients, (3) naïve to ART, and (4) started TDF/FTC/EFV as a first-line regimen. We excluded patients with (1) calcium supplementation greater than 500 mg/day or vitamin D supplementation greater than 800 IU/day, (2) serum calcium of more than 10.5 g/dL, (3) creatinine clearance <60 mL/min/1.73 m^2, (4) any osteoporosis treatment, (5) receiving steroid (equivalent to prednisone >5 mg/day and >3 months), (6) chemotherapy within the past 6 months, (7) clinically active thyroid disease, (8) history of fragility fracture, (9) documented osteoporosis, (10) nephrolithiasis, (11) currently active opportunistic infection, (12) secondary amenorrhea, and (13) pregnant or breastfeeding women.

Patients were randomly assigned in a 1:1 ratio to either the study group, who received 20,000 IU vitamin D_2 weekly [53] plus 1,250 mg of calcium carbonate daily, taken with food for good absorption, or the control group, who did not receive these supplementations. All participants were advised of adequate intake of calcium from food and nonphar-

macologic measures to attenuate bone density loss (*e.g.*, abstinence from smoking and alcohol drinking). Patients were encouraged to perform weight-bearing exercise, resistive exercise, and increase physical activity with the goal of maintaining bone density. Follow-up appointments were scheduled every 12 weeks for 2 visits. Every visit, patients were asked for a self-report on adherence to antiretroviral drugs, vitamin D, and calcium.

BMD assessment was measured at baseline and at week 24. Each subject changed into light clothing before undergoing BMD assessment by dual-energy X-ray absorptiometry (DXA) at the lumbar spine (L1-L4 vertebrae), total hip, and femoral neck [54]. Using fast-array mode, all measurement procedures were performed according to the International Society for Clinical Densitometry (ISCD) recommendations [55] by ISCD-certified densitometry technologists using the same Hologic Discovery DXA scanner on all subjects (Hologic, Bedford, MA). The BMD root mean square (RMS) coefficient of variation (CV) and RMS standard deviation (SD) were, respectively, 0.69% and 0.006 g/cm^2 at the lumbar spine, 1.68% and 0.012 g/cm^2 at the femoral neck, and 0.73% and 0.006 g/cm^2 at the total hip. A Z-score (BMD compared with age-, sex-, and ethnicity-matched reference population) of –2 or less was defined as low bone mass.

Venous whole blood samples were collected into ethylenediaminetetraacetic acid (EDTA). Total 25(OH)D levels (reference range, 30.0-110.0 ng/mL) were measured by liquid chromatography–mass spectrometry (Agilent Technologies, Santa Clara, CA, USA). Samples were deproteinized by protein crash method and loaded onto C18 Guard column (Zorbax Eclipse Plus 2.1x12.5mm, 5 Um, Agilent Technologies) to trap the analytes of interest and directed to analytical column (Poroshell 120 EC-C18, Agilent Technologies) maintained at 50◦C for future separation. MS was operated in multiple reaction monitoring (MRM) mode for specific transitions for vitamin D_2 [25(OH)D_2] and vitamin D_3 [25(OH)D_3], along with a deuterated 25(OH)D_3 as an internal standard, for relative quantification of vitamin D_2 and D_3. Total serum 25(OH)D vitamin level represents the summation of serum 25(OH)D_2 and 25(OH)D_3. The intra-assay CVs of the serum 25(OH)D_2 level and 25(OH)D_3 level were 2.5%-3.0% and 1.7%-3.2%, respectively. The interassay CVs of serum 25(OH)D_2 and serum 25(OH)D_3 levels were 3.2%-3.5% and 2.5%-4.0%, respectively. Vitamin D deficiency was defined as a 25(OH)D level <20 ng/mL, and vitamin D insufficiency was defined as a 25(OH)D level of 21-29 ng/mL. Intact PTH levels (reference range, 15-65 pg/mL) were measured by sandwich immunoassay with electrochemiluminescent detection. Biomarkers including P1NP (reference range: male, 48.0-68.6 ng/mL and premenopause, 15.15-58.59 ng/mL) and CTX (reference range: males, 30-50 years 0.016-0.584 ng/mL and premenopause, 0.025-0.573 ng/mL) were measured by electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN, USA). CVs for all assays were within acceptable limits. Plasma samples at baseline and week 24 were stored at –80°C.

2.2. Statistical Analysis

We calculated that 22 patients per group would provide 80% power to detect a treatment difference of 1.7% in BMD in mean total hip change between groups [33], assuming a standard deviation of 2.3% [56] and a two-sided *T*-test with an α of 0.05. Assuming a 10% dropout rate, we planned total sample size of 48. We performed the analysis with an intention-to-treat approach.

Continuous variables were described as means and SD or median and interquartile range (IQR). Proportions were given for categorical variables. The percentage change of BMD was calculated from BMD at baseline and week 24. To compare variables between the study groups, χ^2 or Fisher's exact test and Wilcoxon rank-sum test were performed. Wilcoxon signed rank test and paired Student's *t*-test were performed to evaluate differences within treatment groups. All analyses were performed by Stata/MP version 14.0 (StataCorp LP, Lakeway Drive College Station, TX, USA).

3. RESULTS

Eighteen participants were enrolled (9 in the study group and 9 in the control group) and were followed up for 24 weeks (Fig. **1**). Baseline demographic data and other variables of eligible patients are summarized (Table **1**). The patients' mean (SD) age was 30.3 (8.9) years and median (IQR) CD4 cell count was 242 (74-287) cells/mm^3. Most of the study population were men who have sex with men (77.8%). No patients had hepatitis C co-infection. The mean (SD) baseline total 25(OH)D level of all patients was 22.2 (6.5) ng/mL. Seven patients had vitamin D deficiency (4 in the study group and 3 in the control group), and 9 patients had vitamin D insufficiency (4 in the study group and 5 in the control group). All parameters were balanced in both groups. All patients completed study follow-up and had 100% adherence to ART as well as vitamin D_2 plus calcium supplement. None of the patients switched their ART regimen during the study's follow-up period.

At baseline, total hip, femoral neck, and lumbar spine BMD were not different between the study and control group (Table **1**). When the BMD results were classified according to the z-score, 2 patients in the control group and none in the study group had low bone mass at baseline. At week 24, total hip BMD significantly decreased from baseline in both groups (Table **2**). However, there was no significant difference in total hip BMD at week 24 between the two groups. Similarly, there was a reduction in lumbar spine BMD in both groups (Table **2**). Of note, patients in the study group had higher lumbar spine BMD at week 24 when compared with those in the control group ($p = 0.042$).

For femoral neck BMD, the changes from baseline and BMD at week 24 were not different (Table **2**) between the two groups. A comparison of the percentage change of BMD between the study group and the control group at each anatomical site showed no statistical significance (Table **3**). From baseline to week 24, median (IQR) percentages of decline in total hip BMD in the study group were –2.7% (–4.9% to –1.6%) and those in the control group were –2.8% (–4.3% to –2.5%; $p = 0.757$). The median (IQR) percentage changes of lumbar spine BMD were –3.6% (–4.4% to –2.5%) in the study group compared with –4.0% (–4.9% to –1.3%) in the control group ($p = 0.825$). The median (IQR) percentage changes of femoral neck BMD were –4.0% (–6.1% to –2.1%) in the study group compared with –0.7%

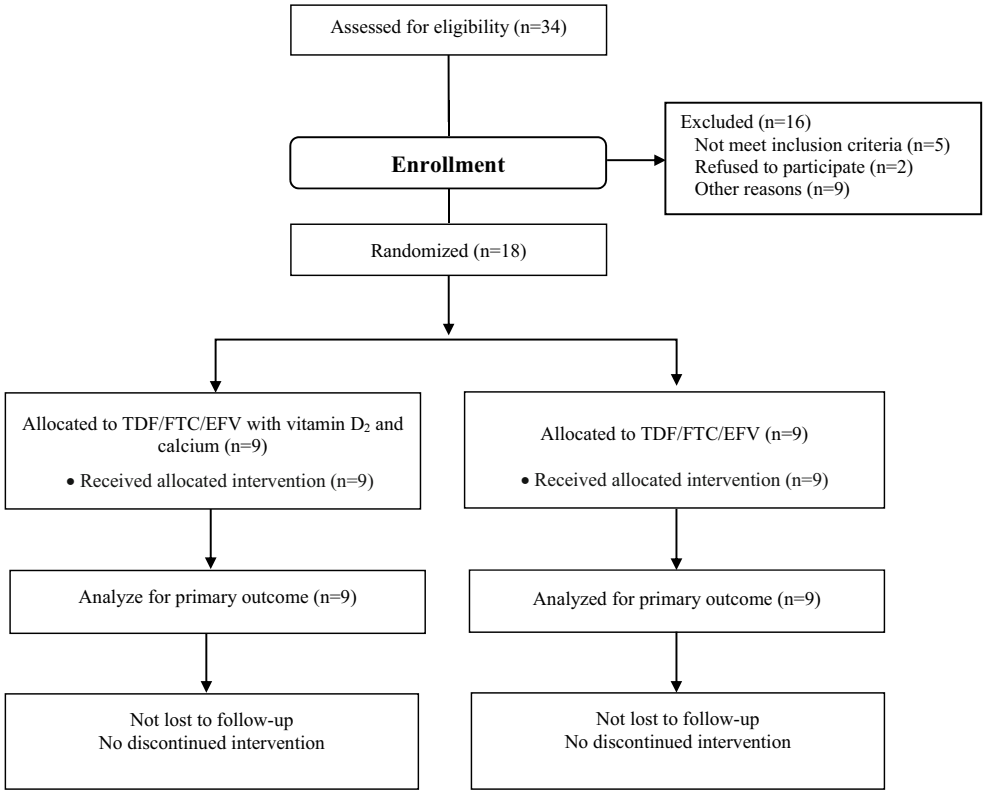

Fig. (1). Study flow diagram.

Table 1. Baseline demographic characteristic of 18 patients.

Variable	All (N = 18)	Vitamin D plus calcium (n = 9)	Control (n = 9)	*P*
Mean (SD) age, years	30.3 (8.9)	31 (7.1)	29.7 (10.8)	0.761
Men, n (%)	17 (94.4)	9 (100)	8 (89.0)	0.303
MSM, n (%)	14 (77.8)	8 (88.9)	6 (66.7)	0.257
Mean (SD) BMI, kg/m^2	21.4 (7.1)	23.8 (9.6)	19.01 (2.7)	0.161
Median (IQR) CD4 cell count, $cells/mm^3$	242 (74- 287)	234 (74-290)	286 (144-286)	0.994
History of smoking, n (%)	3 (16.7)	22 (22.2)	1 (11.1)	0.527
History of substance abuse, n (%)	3 (16.7)	22 (22.2)	1 (11.1)	0.527
History of alcohol drinking, n (%)	6 (33.3)	3 (33.3)	3 (33.3)	1.000
Mean (SD) GFR, mL/ min/1.73 m^2	112.3 (12.9)	114.8 (11.7)	109.9 (14.2)	0.433
Mean (SD) total hip BMD, g/cm^2	0.92 (0.13)	0.96 (0.14)	0.87 (0.11)	0.138
Median (IQR) Z-score for total hip BMD	–0.3 (–0.6 to 0.6)	–0.3 (–0.6 to 1.2)	–0.4 (–0.5 to –0.3)	0.267
Mean (SD) lumbar spine BMD, g/cm^2	0.95 (0.12)	1.00 (0.13)	0.90 (0.09)	0.058
Median (IQR) Z-score for lumbar spine BMD	–0.5 (–1.2 to 0.2)	–0.3 (–0.9 to 0.7)	–0.6 (–1.2 to –0.5)	0.122
Mean (SD) femoral neck BMD, g/cm^2	0.81 (0.14)	0.85 (0.14)	0.77 (0.13)	0.210
Median (IQR) Z-score femoral neck BMD	–0.2 (–0.9 to 0.8)	0 (–0.8 to 1.3)	–0.3 (–1.1 to 0.1)	0.200
Mean (SD) TBS of L1-L4	1.40 (0.07)	1.40 (0.07)	1.40 (0.07)	0.995
Mean (SD) CTX, ng/mL	0.41 (0.22)	0.43 (0.28)	0.40 (1.50)	0.785
Mean (SD) P1NP, ng/mL	49.4 (21.4)	56.5 (21.1)	42.4 (20.1)	0.167
Mean (SD) total 25(OH)D, ng/mL	22.2 (6.5)	21.7 (7.6)	22.8 (5.5)	0.732
Mean (SD) PTH, pg/mL	39.0 (15.6)	40.2 (19.2)	37.7 (12.1)	0.746

Table 2. Summary of bone mineral density, total 25(OH)D level, bone turnover markers, and parathyroid hormone level at baseline and week 24 of 18 patients.

Variable/study Group	Baseline	Week 24	*P*
Mean (SD) total hip BMD, g/cm^2	-	-	0.141$^{\Delta}$
Vitamin D_2 plus calcium	0.96 (0.14)	0.93 (0.13)	0.006¶
Control	0.87 (0.11)	0.84 (0.11)	0.004*
Mean (SD) lumbar spine BMD, g/cm^2	-	-	0.042$^{\Delta}$
Vitamin D_2 plus calcium	1.00 (0.13)	0.97 (0.13)	0.004¶
Control	0.90 (0.09)	0.86 (0.08)	0.006*
Mean (SD) femoral neck BMD, g/cm^2	-	-	0.297$^{\Delta}$
Vitamin D_2 plus calcium	0.85 (0.14)	0.82 (0.17)	0.057¶
Control	0.77 (0.13)	0.74 (0.15)	0.070*
Mean (SD) TBS of L1-L4	-	-	0.879$^{\Delta}$
Vitamin D_2 plus calcium	1.40 (0.08)	1.38 (0.10)	0.317¶
Control	1.40 (0.08)	1.38 (0.08)	0.397*
Mean (SD) total 25(OH)D, ng/mL	-	-	0.436$^{\Delta}$
Vitamin D_2 plus calcium	21.7 (7.6)	30.4 (10.4)	0.030¶
Control	22.8 (5.5)	26.6 (9.9)	0.380*
Mean (SD) P1NP level, ng/mL	-	-	0.408$^{\Delta}$
Vitamin D_2 plus calcium	56.5 (21.1)	90.0 (68.6)	0.157¶
Control	42.4 (20.3)	69.2 (26.1)	0.013*
Mean (SD) CTX level, ng/mL	-	-	0.600$^{\Delta}$
Vitamin D_2 plus calcium	0.43 (0.28)	0.49 (0.47)	0.529¶
Control	0.40 (0.15)	0.40 (0.15)	0.967*
Mean (SD) PTH level, pg/mL	-	-	0.219$^{\Delta}$
Vitamin D_2 plus calcium	40.2 (19.2)	52.2 (25.2)	0.057¶
Control	37.7 (12.1)	40.71 (9.3)	0.584*
Median (IQR) CD4 cell count, cell/mm^3	-	-	0.310$^{\Delta}$
Vitamin D_2 plus calcium	234 (74 to 290)	496 (201 to 649)	0.998¶
Control	249 (144 to 286)	315 (189 to 337)	0.981*
Mean (SD) GFR, mL/min/1.73 m^2	-	-	0.795$^{\Delta}$
Vitamin D_2 plus calcium	114.8 (11.7)	110.7 (12.8)	0.166¶
Control	109.9 (14.2)	109.1 (13.6)	0.768*
Mean (SD) calcium, g/dL	-	-	0.666$^{\Delta}$
Vitamin D_2 plus calcium	9.2 (0.5)	9.4 (0.3)	0.151¶
Control	9.3 (0.5)	9.5 (0.2)	0.379*
Mean (SD) phosphate, g/dL	-	-	0.254$^{\Delta}$
Vitamin D_2 plus calcium	3.7 (0.8)	3.8 (0.6)	0.664¶
Control	3.5 (0.4)	3.5 (0.6)	0.920*

¶ Evaluate the difference in changes from baseline to week 24 within the vitamin D_2 and calcium supplement group.
* Evaluate the difference in changes from baseline to week 24 within the control group.
$^{\Delta}$Evaluate the difference at week 24 between the two groups.

Table 3. Median percentage change of BMD from baseline to week 24 in 18 patients.

Anatomical Site	Percentage Change from Baseline to Week 24		*P*
	Vitamin D_2 Plus Calcium	Control	
Median (IQR) total hip BMD	–2.7% (–4.9% to –1.6%)	–2.8% (–4.3% to –2.5%)	0.757
Median (IQR) lumbar spine BMD	–3.6% (–4.4% to –2.5%)	–4.0% (–4.9% to –1.3%)	0.825
Median (IQR) femoral neck BMD	–4.0% (–6.1% to –2.1%)	–0.7% (–7.2% to –0.2%)	0.825

(–7.2% to –0.2%) in the control group (p = 0.825). According to the z-score, 2 patients in the control group and 1 patient in the study group had low bone mass at week 24.

In the study group, there was a significant increase in mean (SD) total 25(OH)D level from baseline to week 24 (21.7 [7.6] ng/mL vs 30.4 [10.4] ng/mL, p = 0.030). In the control group, there was no significant increase of mean (SD) 25(OH)D level from baseline to week 24 (22.8 [5.5] ng/mL vs 26.6 [9.9] ng/mL, p = 0.380). However, the mean total 25(OH)D levels at week 24 in both groups were not statistically significantly different (p = 0.436). With regard to vitamin D status, 5 patients in the study group and 3 patients in the control group, respectively, had vitamin D sufficiency. There was a trend of an increase in mean (SD) PTH levels from baseline to week 24 in the study group (40.2 [19.2] pg/mL vs 52.2 [25.2] pg/mL, p = 0.057). The difference in mean PTH levels at week 24 did not reach statistical significance between the two groups (p = 0.219). In the control group, a significant change in mean (SD) P1NP from baseline to week 24 was observed (42.4 [20.3] ng/mL vs 69.2 [26.1] ng/mL, p = 0.013). There was no statistically significant difference in mean (SD) CTX level between the study group and the control group at week 24 (0.49 [0.47] ng/mL vs 0.40 [0.15] ng/mL, p = 0.60; Table **2**).

No side effects of vitamin D_2 plus calcium supplement were reported. Mean (SD) calcium levels in the study group at baseline and week 24 were 9.2 (0.5) g/dL and 9.4 (0.3) g/dL, respectively (p = 0.151). There was no significant difference in mean (SD) phosphate levels at baseline and week 24 in the study group, 3.7 (0.8) g/dL and 3.8 (0.6) g/dL, respectively (p = 0.664). No significant decline in glomerular filtration rate (GFR) in the study group was observed. All patients in both groups had virological suppression without significant change in CD4 cell count levels from baseline to week 24.

4. DISCUSSION

In this study of Thai HIV-infected patients, a significant reduction in total hip BMD and lumbar spine BMD, but not femoral neck BMD, was demonstrated as early as 24 weeks after the recent initiation of TDF/FTC/EFV. When compared with participants in the control group, the percentage changes of total hip, lumbar spine, and femoral neck BMD of participants receiving 20,000 IU vitamin D_2 weekly and 1,250 mg of calcium carbonate daily were not different. However, at week 24, the lumbar spine BMD of participants in the study group was higher than those in the control group. Supplementation with 20,000 IU vitamin D_2 weekly for 24 weeks significantly increased total 25(OH)D levels in the study group. Of note, we demonstrated a significant increase in P1NP in the control group but not in the study group. Due to the small sample size and short duration of follow up, we cannot give a conclusion that calcium and vitamin D supplement decrease bone loss in HIV-infected patients in the Thai population.

In ART naïve HIV-infected patients, many studies demonstrated more significant bone loss at both total hip and lumbar spine and higher bone turnover after initiation of TDF than with other NRTIs [5, 28-30]. Similar findings were observed in patients who were on stable and effective ART as well as in those who switched other antiretroviral agents to TDF-containing regimens [6, 57, 58]. Even though our study had a follow-up period of only 24 weeks, we demonstrated a significant change in total hip and lumbar spine BMD in both study groups, who were young adult patients. The percentage change in total hip BMD was –2.8% in the control group and –2.7% in the study group at week 24. The magnitude of bone loss in our patients was similar to that of postmenopausal women, despite the fact that most of the population in our study were young males. This raises a concern of increased fracture risk from ART in HIV-infected individuals, in which TDF/FTC/EFV is still the most frequently used regimen, especially in Thailand and many resource-limited settings, according to the WHO guidelines [59].

Despite the significant increase in total 25(OH)D levels after 24 weeks of vitamin D_2 supplementation in the study group, BMD at all sites was not different among the study and control groups. However, greater lumbar spine BMD in the study group compared with the control group at week 24 was observed. Our result was different from a study in the US population by Overton et al, who demonstrated an effect of vitamin D_3 and calcium supplement in attenuating bone loss of total hip rather than lumbar spine BMD in ART naïve HIV-infected patients who initiated TDF/FTC/EFV [33]. In other studies, in which vitamin D_3 was given to adults with HIV infection who were treated TDF, lumbar spine BMD but not total hip BMD was increased in the vitamin D group over 48 weeks [60, 61]. In addition, an increase in lumbar spine BMD was also observed in HIV-infected individuals who received vitamin D_3 and calcium regardless of ART regimen [62]. The longitudinal study in Thai HIV-infected adolescents with low bone mass who calcium and vitamin D_3 were given. After a 6-month follow-up, the median lumbar spine BMD increased significantly, however this study did not measure BMD at the total hip [63]. Of note, different forms of vitamin D supplementation (vitamin D_2 vs vitamin

D_3) could partly explain the difference in these results. Of note, we supplemented vitamin D_2 in the study group since vitamin D_2 is the only pharmacological form available in Thailand. Similar to other studies on the effect of vitamin D and calcium supplementation in bone health in the general population, the difference in patient characteristics (*e.g.*, ethnicity, gender, body mass index, and baseline 25[OH]D levels) and study design influence the differences in the results between our study and others. The dosage of calcium supplement also needed to be discussed. The study conducted in 1999 showed low dietary intake of calcium in Bangkok metropolitan was 366.5 mg in male and 286.7 mg in female [64]. Current data suggests greater of Thai dietary calcium intake is 672 mg [65]. Supplementation with calcium carbonate 1,250 mg (equal to elemental calcium 500 mg) was adequate without an adverse event.

Compared with other drugs, TDF was associated with a more significant increase in bone markers [5, 40, 66, 67]. In our study, there was no difference in the change in CTX from baseline in both groups. Interestingly, P1NP was significantly increased from baseline to week 24 only in the control group. This result was similar to the findings of a previous study that demonstrated a reduced change in bone markers in participants receiving vitamin D and calcium supplementation [68].

Almost 90% of our patients had vitamin D deficiency and insufficiency at baseline, a value that is comparable to those in some cohorts [10, 37, 39]. Risk factors for low vitamin D are current smoking, low CD4 cell count, higher GFR, and antiretroviral drug use [69, 70]. The only antiretroviral drug associated with vitamin D efficiency was EFV [10, 37-39]. Mechanistically, EFV is an inducer of CYP450 D-24-hydroxylase (CYP24A) that catalyzes the breakdown of active vitamin D ($1,25[OH]_2D$ and 25[OH]D) into inactive metabolites [71]. In our cohort, 16.7% of the participants were current smokers, and 38.8% of the participants had a CD4 cell count less than 200 cells/mm^3.

The initiation of ART containing TDF was associated with elevation of PTH levels which were greater among those with vitamin D deficiency [72-74]. This might suggest the possibility that the upregulation of PTH plays a role in the decrease of BMD [72]. In patients with stable ART, there was an association between higher plasma vitamin D binding protein and lower free vitamin levels with TDF use [75]. This functional vitamin D deficiency may cause secondary hyperparathyroidism [36]. A recent study reported no significant change in PTH levels in individuals with vitamin D_3 and calcium supplementation, but there was a significant increase in PTH levels in the placebo group [33]. In addition, the between-group differences in PTH levels were statistically significant at week 24 and week 48 [33]. In our study, there was a trend of an increase in PTH levels at week 24 compared with baseline in the study group ($p = 0.057$). The increase in PTH levels from the baseline in the study group might have been influenced by 2 participants who had high PTH levels, 93.1 and 84.0 pg/mL. Both had normal calcium, phosphorus, and total 25(OH)D levels as well as normal renal function. This was suspected to be a condition called normocalcemic primary hyperparathyroidism, which is characterized by normal serum calcium and elevated PTH levels in the absence of vitamin D deficiency, renal disease, or medications, which elevate PTH levels. However, after excluding these two patients, there was no statistically significant difference in mean (SD) PTH levels from baseline to week 24 in the study group (32.34 [10.34] pg/mL vs 41.67 [16.25] pg/mL, P = 0.068).

The strength of this study is that it was conducted in Thai HIV-infected individuals. Then the results could be employed in general practice in patients of similar ethnicities (*i.e.*, Asian) and geographic regions (*i.e.*, tropical setting) where EFV/TDF/FTC is still a relevant first-line drug in Thailand and some other countries. A significant change in BMD as early as 24 weeks of initiated ART was shown. This study demonstrated the use of vitamin D_2 for supplementation instead of vitamin D_3, since vitamin D_3 is not available in many countries. As a matter of fact, vitamin D_3 raises the total 25(OH)D level better than vitamin D_2 does; however, the study in Thailand showed that vitamin D_2 20,000 IU weekly was adequate to raise total 25(OH)D levels [53]. It remains to be determined whether interventions to alleviate bone loss during this period result in a difference in fracture risk.

This study had several limitations, including a small sample size, which may cause underpowered to demonstrate the difference of BMD change between the study groups. We had a limited time of enrollment and performance in a single center. The low expected rate of new infections with a predefined short timeline for inclusion leads to the low number of subjects enrolled rather than other factors. Because of the short duration of follow-up, we could not evaluate the long-term effect of vitamin D_2 and calcium supplements, for example, decreased bone fracture. Most study patients were male and men who have sex with men; thus, the results may be difficult to generalize to other populations, such as females and HIV-infected individuals with heterosexual risk.

CONCLUSION

Vitamin D_2 and calcium supplementation may provide some benefit in attenuating bone loss in HIV-infected patients who received TDF/FTC/EFV. A long-term study with a larger sample size should be conducted to explore the effect of vitamin D and calcium supplementation in this population.

LIST OF ABBREVIATIONS

25(OH)D	=	25-hydroxyvitamin D
3TC	=	Lamivudine
ABC	=	Abacavir
ART	=	Antiretroviral Therapy
BMD	=	Bone Mineral Density
CTX	=	C-Terminal Cross-Linking Telopeptide of Type 1 Collagen
CV	=	Coefficient of Variation
CYP24A	=	CYP450 D-24-Hydroxylase
DXA	=	Dual-Energy X-Ray Absorptiometry

EFV	=	Efavirenz
FTC	=	Emtricitabine
GFR	=	Glomerular Filtration Rate
IQR	=	Interquartile Range
ISCD	=	International Society for Clinical Densitometry
NNRTI	=	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	=	Nucleoside Reverse Transcriptase Inhibitors
PN1P	=	Procollagen Type 1 Amino-Terminal Propeptide
PTH	=	Parathyroid Hormone
SD	=	Standard Deviation
TAF	=	Tenofovir Alafenamide
TBS	=	Trabecular Bone Score
TDF	=	Tenofovir Disoproxil Fumarate
WHO	=	World Health Organization

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approvals of this study were obtained from the committee on human rights related to research involving human subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (Approval No. ID 01-59-11).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

All patients provided written informed consent prior to participation in the study.

STANDARD OF REPORTING

This study has followed the CONSORT guidelines for RCT.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

No competing financial interests exist. Parts of this manuscript were presented at the European Congress of Clinical Microbiology and Infectious Diseases (Madrid), April 21-24, 2018 (abstract P1929).

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