

Studies on Vitamin D Levels in Serum of HIV Infected Patients: Their Effect on Progression towards AIDS

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Abstract

Vitamin D deficiency may be more prevalent among HIV-positive patients than in the general population due to HIV disease-related factors. This study examined the effects of HIV infection and use of antiretroviral drugs in serum vitamin D levels in HIV patients visiting Aga Khan University Hospital, Nairobi Kenya from October 2013 to April 2014. The effect of vitamin D status on CD₄ cell count and HIV viral load was evaluated to determine the status of disease progression to AIDS. HIV viral load in blood samples was determined using COBAS Ampliprep/TaqMan HIV-1 test kit while CD₄ cell counts were done using the fluorescence-activated cell sorter system. The levels of vitamin D in serum were determined using electrochemiluminescence binding assay in Cobas E601 mass analyzers. In addition, selected plasma enzymes were used to evaluate liver function. Higher percentage (49.12%) of deficient vitamin D cases were observed among HIV patients not on ART. Deficient levels of Vitamin D were associated with abnormal selected liver enzymes. High viral load was observed among patients not on ART with deficient and insufficient vitamin D. The CD₄ cell count was higher in patients on ART with sufficient vitamin D levels compared to those with deficient vitamin D. These observations suggest a need to supplement ART with vitamin D in order to ameliorate Vitamin D deficiency as a strategy to improve HIV management.

Keywords

Vitamin D, HIV Patients, ART, Viral Load, CD₄ Count

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1. Introduction

Human Immunodeficiency Virus (HIV) infection is characterized by a progressive deterioration in immune function. Interventions that offset this impairment have the potential to slow HIV disease progression and improve quality of life [1]. Vitamin D may represent one such intervention because of its involvement in the human immune response. Although the mechanisms implicated are not fully understood, laboratory studies have suggested an important role of vitamin D in immune regulation; the discovery of vitamin D receptors (VDRs) on peripheral blood mononuclear cells has fuelled the interest in vitamin D as an immune modulator. More recently, vitamin D has been shown to have an integral role in the innate immune response to infections such as tuberculosis [2].

The relationship between vitamin D and HIV disease progression has become a topic of interest to research. A study in Norway found that HIV-infected patients with low 25-dihydroxyvitamin D₃ levels, the biologically active metabolite of vitamin D, had significantly shorter survival time than those with normal concentrations [3]. In another study in Tanzania, HIV-infected pregnant women were supplemented with multivitamins lacking vitamin D and followed up to observe pregnancy outcomes and disease progression. A correlation was drawn between reduced levels of Vitamin D and the rate of HIV disease progression to AIDS [4].

Although there is no agreement among international experts on the most appropriate cut-off value for adequate vitamin D level, individuals with 25-(OH)D below 20 ng/ml are considered as deficient [2]. However, a value above 30 ng/ml has been suggested to associate with better health outcomes such as higher bone mineral density, less falls and fractures as well as protection against cancer in the general population [2]. A low vitamin D level has recently been associated with increased mortality in the general population [5] as well as with HIV disease progression and overall mortality in a cohort of Tanzanian pregnant women with HIV infection [1].

Patients receiving anti-retroviral (ARV) therapies are at a greater risk for developing vitamin D deficiency and are at higher risk of developing bone problems than patients not taking ARV treatments [6] [7].

It has also been observed that ART may be affecting normal levels of vitamin D, parathyroid hormone (PTH), and BMD, significantly increasing risk of developing fractures [8]. Measurement of vitamin D in serum and supplementation of the vitamin in those with low levels has been widely adopted in HIV clinical practice. However, not much data on the effect of vitamin D on CD₄ cell count and viral load is available. Also, information on the clinical benefits and cost effectiveness of vitamin D supplementation is scarce. This study therefore examined the relationship between HIV infection, ART and levels of serum vitamin D; their effects on CD₄ cell count and viral load in HIV positive patients.

2. Materials and Methods

2.1. Informed Consent

All participants in the study enrolled willingly through informed consent. Participant information was handled with utmost confidentiality and all ethical clearance for the study was assented to by the Aga Khan University Hospital Scientific and Ethical Review.

2.2. Sample Size

118 HIV positive blood samples were evaluated during the study period. This sample size was purposively determined taking into consideration the patient presentation at the study site.

2.3. Collection of Blood Samples

All samples used in this study were obtained with ethical approval of the institution. Five milliliters of venous blood from consenting patients was drawn into EDTA vacutainer tube with clot activator. The samples were centrifuged in a table top high speed capacity centrifuge at 5000 g for 5 min to obtain serum. The plasma samples were coded and stored for further analysis. Another five milliliters was drawn into a clean EDTA (liquid) containing vacutainer tube for the analyses of CD₄ cell count.

2.4. Determination of Serum Vitamin D

The vitamin D assay was performed using electrochemiluminescence binding assay in Cobas E601 mass ana-

lyzer (Roche Diagnostics). The levels of vitamin D were expressed in nanograms per milliliter.

2.5. CD₄ Count

A FACS Count fluorescence-activated cell sorter (FACS) system (Becton Dickinson) was used to enumerate absolute values for CD₄ cells in unlysed whole blood containing EDTA. This blood was added to ready-to-use reagent containing tubes for the determination of the absolute counts of CD₄ + T lymphocytes according to the manufacturer's instructions. The CD₄ was expressed in copies per milliliter (cp/ml).

2.6. Determination of HIV-1 Viral Load

The HIV-1 RNA in plasma was quantitated using COBAS Ampliprep/COBAS TaqMan HIV-1 test version 2.0. The samples were processed in COBAS Ampliprep system and quantitated using COBAS TaqMan analyzer. The viral load was expressed in copies per milliliter.

2.7. Assay of Enzymes Associated with Liver Function

Alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and Alanine amino transferase (ALT) enzymes were analysed using the enzyme calorimetric assay in accordance with the standardized method recognized by international federation of clinical chemistry (IFCC). The analysis was conducted using cobas e601 mass analyzer. The quantity of enzyme present in plasma was expressed in units per liter (units/liter).

3. Results

3.1. Status of Serum Vitamin D in HIV Positive Patients

Serum vitamin D was determined in 118 patients. Fifty seven (57) of the participants were HIV infected and not receiving antiretroviral therapy and sixty one (61) were HIV infected and receiving antiretroviral therapy. Overall, 46 (39%) of the patients were vitamin D deficient (≤ 20 ng/ml), 40 (34%) were vitamin D insufficient (21 - 29 ng/ml) and 32 (27%) were vitamin D sufficient (≥ 30 ng/ml). Out of the fifty seven patients not receiving treatment, 28 (49.12%) were vitamin D deficient, 20 (35.09%) were vitamin D insufficient and 9 (15.79%) had vitamin D sufficient. Out of 61 patients who were on treatment 18 (29.51%) were vitamin D deficient, 20 (32.78%) were vitamin D insufficient and 23 (37.71%) were vitamin D sufficient (**Table 1**). None of the participants were reported to have vitamin D hypervitaminosis (>74 ng/ml).

3.2. CD₄ Cell Count

Amongst patients not on ART and vitamin D deficient 70.4% had CD₄ cell count < 200 cp/ml, 25.9% had CD₄ count between 200 - 499 cp/ml and 3.7% had CD₄ count ≥ 500 cp/ml. In patients with sufficient vitamin D and not on ART 44.4% of the patients had CD₄ cell count < 200 cp/ml, 55.6% had CD₄ cell count between 200 - 499 cp/ml and none of the patient had CD₄ count ≥ 500 . Patient who were HIV+ART+ with deficient vitamin D had 44.4% of the patients with CD₄ count < 200 cp/ml, 27.7% with CD₄ cell count between 200 - 499 cp/ml and

Table 1. Status of vitamin D in serum of HIV positive patients.

	Vitamin D levels			
	Total	Deficient (≥ 20 ng/ml)	Insufficient (21 - 29 ng/ml)	Sufficient (≥ 30 ng/ml)
		No. (%) of patient		
N	118	46 (38.98)	40 (33.90)	32 (27.11)
Patient type				
HIV positive not on ART	57	28 (49.12)	20 (35.09)	9 (15.79)
HIV positive on ART	61	18 (29.51)	20 (32.78)	23 (37.71)

n—Number of participants. ART indicates antiretroviral therapy; HIV+ indicates HIV infected participants. Vitamin D deficiency was proportionately higher in patients who were not on ART than in patients who were on ART.

27.7% of the patients had CD₄ count \geq 500 cp/ml compared to patients with sufficient vitamin D 30.4% had CD₄ count $<$ 200 cp/ml, 26.1% had CD₄ cell count between 200 - 499 cp/ml and 43.5% had CD₄ count \geq 500 cp/ml (**Table 2**).

3.3. Determination of Viral Load

HIV viral load was defined as low when the levels were $<$ 10,000 cp/ml, moderate when the levels were 10,000 - 100,000 cp/ml and high when the levels were $>$ 100,000 cp/ml. In HIV patients who were not on treatment and were deficient in vitamin D, those with low viral load contributed to 21.1%, those with moderate viral load formed 31% while those with high viral load formed 65.2%. In the same group, patients with sufficient vitamin D were observed to have low viral load in 37.5%, moderate viral load in 25% and high viral load in 37.5%. Results obtained from HIV+ART+ showed that patients with deficient Vitamin D formed 61.1% of the patients with low viral load, 16.7% had moderate viral load and 22.2% had high viral load. Patients with sufficient vitamin D and on ART were observed to have low viral load in 77.3%, moderate viral load in 4.5% and high viral load in 18.2% as shown in **Table 2**.

3.4. Levels of Selected Serum Enzymes Associated with Liver Function

In HIV+ART-, ALP levels in patients with vitamin D deficiency were normal in 25% and abnormal in 75%, GGT levels were normal in 28.57% and abnormal in 71.42% and ALT levels were normal in 57.42% and abnormal in 42.85%. Patients with sufficient vitamin D and not on ART had ALP levels normal in 8 (88.89%) and abnormal in 1 (11.11%). GGT levels were normal in 7 (77.78%) and abnormal in 2 (22.28%). ALT levels were normal in all the patients. In HIV+ART+ patients with deficient vitamin D ALP levels were normal in 72.22% and elevated in 27.78%. GGT was normal in 72.22% and elevated in 27.78%. ALT was normal in 88.89% and elevated in 11.11%. In HIV+ART+ patients who had sufficient vitamin D levels, ALP levels were normal in 60.87% and abnormal in 39.13% patients. GGT levels were normal in 60.87% and abnormal in 39.13% patients. ALT was normal in 78.26% and abnormal in 21.74% of the patients (**Table 3**).

4. Discussion

The percentage of patients with sufficient vitamin D was 37.71% in the HIV infected ART receiving group and 15.79% in the group not on ART. In contrast the percentage of patients with deficient vitamin D in the HIV+ART+ group was 29.51% and 49.12% in HIV+ART- group (**Table 1**). This suggests an influence of ART on serum levels of 25(OH) D and 1,25(OH)₂ D. Experimental studies have suggested an inhibitory effect of

Table 2. Distribution of CD₄ cell count and HIV viral load in patients with deficient, insufficient and sufficient vitamin D levels, on ART and not on ART.

	HIV+ART-			HIV+ART+		
	Vitamin D levels					
	Deficient	Insufficient	Sufficient	Deficient	Insufficient	Sufficient
CD₄ T cell category (cp/ml)	No. (% of patients)					
<200	19 (70.4)	9 (50)	4 (44.4)	8 (44.4)	6 (30)	7 (30.4)
200 - 499	7 (25.9)	7 (38.9)	5 (55.6)	5 (27.7)	7 (35)	6 (26.1)
\geq 500	1 (3.7)	2 (11.1)	0 (0)	5 (27.7)	7 (35)	10 (43.5)
HIV viral load (cp/ml)						
<10,000	5 (21.7)	2 (13.3)	3 (37.5)	11 (61.1)	13 (65)	17 (77.3)
10,000 - 100,000	3 (13)	3 (20)	2 (25)	3 (16.7)	2 (10)	1 (4.5)
>100,000	15 (65.2)	10 (66.7)	3 (37.5)	4 (22.2)	5 (25)	4 (18.2)

High percentage of HIV positive patients with deficient vitamin D had low CD₄ cell count with high viral load.

Table 3. Levels of selected enzymes in serum HIV positive patients not on ART and on ART.

Vitamin D levels		HIV+ not on ART			HIV+ on ART		
		Deficient	Insufficient	Sufficient	Deficient	Insufficient	Sufficient
Serum enzymes associated with liver function		No. (% of patients)					
Alkaline phosphatase (units/l)	N	7 (25)	14 (73.68)	8 (88.89)	13 (72.22)	18 (100)	14 (60.87)
	A	21 (75)	5 (26.32)	1 (11.11)	5 (27.78)	0 (0)	9 (39.13)
Gamma glutamyl transferase (units/l)	N	8 (28.57)	12 (63.16)	7 (77.78)	13 (72.22)	11 (61.11)	14 (60.87)
	A	20 (71.42)	7 (36.84)	2 (22.22)	5 (27.78)	7 (38.89)	9 (39.13)
Alanine amino transferase (units/l)	N	16 (57.42)	17 (89.47)	9 (100)	16 (88.89)	17 (94.44)	18 (78.26)
	A	12 (42.85)	2 (10.53)	0 (0)	2 (11.11)	1 (5.56)	5 (21.74)

N—Normal levels; A—Abnormal levels; High percentages of Patients with deficient vitamin D were found to have altered levels of serum enzymes associated with the liver compared to those with sufficient vitamin D.

protease inhibitors on 25(OH) D and 1,25(OH)₂ D synthesis and some cross-sectional studies have found HAART to be associated with lower 25(OH) D while others have found higher 25(OH) D. One such study has shown an association between efavirenz, but not other antiretroviral medication was associated with vitamin D deficiency [9]. Another study showed that efavirenz and zidovudine were associated with vitamin D deficiency. In addition the study participants showed an increase in vitamin D levels when they were changed to a regime boosted with aarunavil with greatest increase among patients changed from efavirenz or zidovudine [10]. The mechanism of efavirenz effect on vitamin D has been linked to induction of Cytochrome P450 like CYP24 which converts both 25(OH) vitamin D and the active form of vitamin D, 1,25(OH) vitamin D to inactive metabolites [11] Efavirenz has been observed to reduce the expression of Cytochrome CYP2R1 which hydroxylates D₃ and D₂ necessary for vitamin D activation [12]. Another study has shown tenofovir to be associated with higher levels of 25(OH) D as it causes proximal tubule injury, inducing renal dysfunction [13] [14]. Given that conversion of 25(OH) D to 1,25(OH)₂ D by 1 α hydroxylase occurs primarily in the proximal tubule, tenofovir-induced proximal tubule dysfunction might also reduce hydroxylation of 25(OH) D leading to normal levels of 25(OH) D and abnormal levels of 1,25(OH)₂ D.

High CD₄ counts (≥ 500 cp/ml) were observed in 36.07% of patients on ART which was more than in patients not on ART (5.56%). The latter had 40.74% of the patients with CD₄ count ≤ 200 cp/ml. levels of serum vitamin D seemed to have an effect on the counts of CD₄ in that 43.5% of patients with sufficient vitamin D levels and on ART had high CD₄ count whereas 44.4% of those with deficient vitamin D had low CD₄ count (≤ 200 cp/ml). this was also observed in patients not on ART. This strongly suggests that vitamin D levels to a greater extent boosts the CD₄ cell counts in both patients on ART and those not on ART. This is in agreement with the findings of Ross *et al.*, de Luis *et al.* and Kim *et al.* who found a positive association between vitamin D with CD₄ count [15]-[17].

The viral load was observed to be significantly higher in patients who were not on ART than in patients who were on ART, with majority of the former having viral loads of $>100,000$ cp/ml and majority of the latter having viral loads of $<10,000$ cp/ml (Table 2). For both groups of patients, a high percentage of patients with sufficient vitamin D levels (37.5% for HIV+ART- and 77.3% for HIV+ART+) were observed to have low viral loads. A high percentage of patients with deficient vitamin D levels in patients not on ART were observed to have high viral load that is 65.2%. Our observations suggest possible role of vitamin D together with ART in lessening the viral load. The role of vitamin D could be explained in the activation of cathelicidin, an antimicrobial peptide found in the lysosomes of macrophages and the secondary granules of neutrophils and can be produced by epithelial cells [18] [19]. Vitamin D up regulates genetic expression of cathelicidin which exhibits broad spectrum microbicidal activity against bacteria, Fungi and viruses [20] In humans only one cathelicidin LL-37 has been identified. Studies have demonstrated that LL-37 has anti-HIV activities with 50% effective concentration for inhibition of viral replication [21] [22]. This may explain why patients with low vitamin D levels were found to have a high viral load. This is consistent with the findings of Kim *et al.* who found that detectable high viremia

was significantly associated with vitamin D deficiency [17].

The effect of vitamin D on progression towards AIDs may be explained by its role in innate and adaptive immunity. The innate immune system is the first line of defense against infections and comprises of innate immune cells like the natural killer cells that have the ability to destroy cells infected by viruses. The innate immune system also recruits immune cells to the sites of infection through the production of cytokines by dendritic cells. Studies have linked low levels of vitamin D with increased infection. A cross sectional study involving 19,000 subjects conducted showed that individuals with low vitamin D levels (<30 ng/ml) were more likely to have an upper respiratory tract infection than those with sufficient levels [23]. Another cross sectional study in Finland involving military recruits showed that those with high vitamin D levels were found to be off duty less days due to upper respiratory tract infections than recruits with low vitamin D levels [24].

Few studies on vitamin D status in HIV related health effects have been conducted but a recent study [25] on the relationship between vitamin D, lipids, HIV infection and HIV treatment showed that total serum 25(OH) D was high in the HIV infected ART treated group compared to untreated HIV group. This is consistent with our findings with the untreated group having low levels of vitamin D (mean 20.181 ng/ml) compared to the treated group (mean 27.619 ng/ml). Mehta *et al* reported that progression to HIV disease stage three as defined by the WHO, was significantly associated with low vitamin D levels. He also reported that low vitamin D levels pose higher risk of developing severe anemia in pregnant women [1] These studies show that vitamin D has a role in HIV disease progression probably linked to its role in innate immunity where it has been shown to improve phagocytic capacity of macrophages, cell mediated immunity and increase natural killer cell number and cytolytic activity. Rook *et al.* reported that hydroxylated metabolites of vitamin D₃ can cause inhibition of the growth of *Mycobacterium tuberculosis* (*M. tuberculosis*) in normal human monocytes [26]. This could explain why vitamin D could be used to prevent progression towards AIDS as *M. tuberculosis* is one of the leading causes of disease progression and mortality in HIV infected patients and increased resistance to tuberculosis could potentially prolong survival in these patients.

In patients not on ART and with sufficient vitamin D, 88.89% cases were found to have normal levels of serum Alkaline phosphatase, 77.78% had normal gamma Glutamyl transferase and all the patients had normal Alanine amino transferase while those with deficient levels of vitamin D were observed to strongly associate with abnormal levels of these enzymes in serum with 75% having abnormal ALP, 71.42% with abnormal GGT and 42.85% with abnormal ALT. This shows that sufficient vitamin D levels in serum associate with high incidence of normal enzymes while deficient levels of vitamin D were observed to strongly associate with abnormal levels of these enzymes in serum. However, this trend was not observed for patients on ART with relatively lower percentages of patients with abnormal levels of these enzymes being observed in this group of patients. This may be explained by the involvement of the liver in vitamin D metabolism given that hydroxylation of vitamin D occurs in the liver and damage to the liver during HIV infection may result in low levels of vitamin D Our study agrees with the findings of a study in Mexico which showed a moderately strong positive correlation between elevated transaminases and HIV RNA in individuals not receiving ART and without viral hepatitis co-infection [27] Consistent with reports from a study in North America [28]. For low and middle income countries, it has been demonstrated that use of ART in individuals with low CD₄ count was associated with a reduced risk of hospitalization for liver related complications [29]. This findings need to be investigated further to determine the causes of the serum enzymes abnormalities in HIV infected patients.

The study also had several limitations. First 25(OH) D was measured at a single time point and we are unable to determine whether deficient vitamin D levels at a single time point or long term deficiency is biologically relevant. Secondly the results of this study were only done in a single hospital and this result may not be generalized to other HIV infected populations and therefore a study including other centres should be conducted to confirm these results. Thirdly neither the daily intake of vitamin D and calcium nor other lifestyle factors such as smoking which could affect vitamin D levels were assessed.

5. Conclusion

High percentages of cases with vitamin D deficiency were observed in HIV-positive patients that were not on ART. A direct positive association is also observed between levels of serum Vitamin D levels with HIV viral load and this suggests that maintenance of sufficient levels of vitamin D in HIV patients along with ART is important in maintenance of high CD₄ cell counts and low viral load. Similarly, low indications of liver dysfunc-

tion were observed in patients with sufficient vitamin D levels more so for those patients on ART. Introduction of Vitamin D supplements with ART should be considered and evaluated in the management strategies for HIV.

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