

# Vitamin D status in Well-Controlled Caucasian HIV Patients in Relation to Inflammatory and Metabolic Markers – A Cross-Sectional Cohort Study in Sweden

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## Abstract

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To study vitamin D (25OH D<sub>3</sub>) in relation to (i) microbial translocation (ii) systemic inflammation and (iii) blood lipid markers, in Caucasian, well-controlled HIV patients and healthy controls, plasma and serum samples from  $n = 97$  male, HIV patients on HAART with immeasurable viral load (<20 copies/ml) since median 6.5 years and no concurrent inflammatory or infectious disease and  $n = 30$  healthy controls were analysed for (i) LPS; (ii) sCD14, hsCRP, IL-4, IL-6, IL-10, IL-17, MCP-1 and IFN- $\gamma$ ; as well as (iii) blood lipids. Vitamin D levels were similarly distributed and equally low in both HIV patients and controls. There was no association between vitamin D levels and markers of microbial translocation, systemic inflammation or dyslipidemia. LPS levels were similar in both groups but HIV patients expressed higher levels of sCD14 and hsCRP, with HIV as an independent risk factor. HIV patients had higher cholesterol and Apo B levels. Notably, more HIV patients smoked and smoking was associated with lower vitamin D levels. In conclusion; these well-treated Caucasian HIV patients had similar vitamin D levels as healthy controls. However, despite perfect virological control, they exhibited slightly increased inflammatory markers and disturbed blood lipids. However, neither of these parameters were associated with low vitamin D levels but appeared to be linked to the HIV-disease *per se*. Thus, the rationale for vitamin D substitution as a way to improve microbial translocation and systemic inflammation is not fully supported in this HIV population.

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## Introduction

Primary HIV infection causes massive damage to lymphoid and mucosal tissues leading to microbial translocation (MT) across a defective gut mucosa and progressive immunodeficiency. Highly active antiretroviral treatment (HAART), effectively suppresses viral replication. However, despite effective treatment, chronic HIV infection is still associated with a persistent systemic inflammation where activation of monocytes and hypercoagulation contribute to premature ageing of the immune system with subsequent vascular disease, metabolic disease and multimorbidity [1]. The central dogma is that low-grade MT is a main driver behind this inflammatory reaction and its associated diseases [2]. A high degree of MT, shown by elevated levels of lipopolysaccharide (LPS) in HIV patients, is associated with increased morbidity and mortality [3]. Likewise, inflammatory markers, such as soluble CD14 (sCD14),

high-sensitivity CRP (hsCRP) and IL-6, are predictors of mortality in HIV patients [4–8].

In recent years, several epidemiological studies demonstrate that also vitamin D deficiency (defined as 25OH D<sub>3</sub> <10 ng/ml, or <30 nmol/l) is associated with disease progression and all-cause mortality in HAART treated [8–12], and treatment-naïve HIV patients [13]. Vitamin D deficiency is associated with cardiovascular disease [14–18], insulin resistance and type-2 diabetes [19–22], in the general population as well as in HIV populations. In HIV patients, vitamin D deficiency is common and established risk factors for low vitamin D levels are as follows: dark skin [23], old age [9], high BMI [23] and ongoing antiretroviral treatment. In particular, Efavirenz treatment has been associated with low vitamin D levels [23–25].

It has been proposed that vitamin D is a key regulator of the barrier function and the inflammatory milieu in the gut. Experimental data have shown that vitamin D promotes gut epithelial differentiation and tight junction

formation [26, 27] and induces expression of antimicrobial peptides [28–30]. Clinical support of this assumption is found in the association between vitamin D deficiency, reduced expression of vitamin D receptor (VDR) [31] and intestinal inflammation [32] in colonic mucosa of patients with inflammatory bowel disease. In mice, targeted expression of human vitamin D receptor (VDR) in intestinal epithelial cells protects from experimental colitis [31].

Given the evidence from observational and experimental studies for a protective role of vitamin D in mucosal immunity it is reasonable to hypothesize that vitamin D may reduce markers for MT, ameliorate systemic inflammation and have a beneficial effect on blood lipids in HIV patients. However, published randomized and placebo-controlled trials (RCTs) on vitamin D's effect on cellular immune response, metabolic and vascular function in HIV patients are inconclusive [33–36]. To our knowledge, there is no study of vitamin D supplementation where reduction of MT and systemic inflammation has been defined as primary endpoints.

To prepare for such a future interventional study, we designed a cross-sectional, observational study where vitamin D levels would be studied in relation to (i) markers for MT (ii) systemic inflammation and (iii) blood lipid markers in a Caucasian, well-controlled group of HIV patients and matched controls. The aim was to collect baseline data to design an interventional study with enough power to test the initial hypothesis.

## Methods

*Design of the study.* Well-controlled (Ongoing HAART and <20 viral copies/ml since 6 months) Caucasian HIV patients ( $n = 97$  males) and controls ( $n = 30$  males) were recruited to study 25-hydroxy vitamin D<sub>3</sub> (25OH D<sub>3</sub>) levels and associations with (i) systemic endotoxin levels (LPS), (ii) systemic inflammation measured by the macrophage activation marker sCD14, as well as the cytokines IL-4, IL-6, IL-10, IL-17, MCP-1, IFN- $\gamma$ , hsCRP and (iii) blood lipids. Faecal calprotectin (f-calprotectin) was measured as a marker of gastrointestinal inflammation. Information on smoking, weight, length and treatment with statins and vitamin D was recorded. Clinical data on HIV patient were collected from patient journals.

*Patient recruitment.* HIV patients were enrolled from the HIV outpatient clinic (Venhälsan) at South General Hospital, Stockholm during a routine clinical visit. Inclusion criteria were as follows: Caucasian origin, age 18–60 years. Exclusion criteria were as follows: Inflammatory bowel disease or other chronic inflammatory disease, ongoing intestinal infection, malignancy, co-infection with tuberculosis, Hepatitis B or C, or ongoing immunosuppressive medication. Male, non-HIV controls were recruited from staff at Karolinska University Hospital.

Patient enrolment took place from January to December 2012. The study protocol was approved by the regional ethics committee of Stockholm (EPN 2011/1383-31/3).

*Laboratory assessment.* Soluble CD14 (sCD14) was measured by enzyme-linked immunosorbent assay (Quantikine<sup>®</sup>, R&D System, Abingdon OX, UK), detectable range 250–16000 pg/ml, coefficient of variation (CV) 4–7%. Lipopolysaccharide (LPS) levels were assessed by chromogenic limulus amoebocyte lysate assay (Lonza, Walkersville, MD, USA), detectable range 0.5–500 pg/ml. Both analyses were performed according to the manufacturer's instructions on EDTA-plasma with a dilution factor of 1:600 and 1:5, respectively. IL-4, IL-6, IL-10, IL-17, MCP-1 and IFN- $\gamma$  were measured in serum by bead-based multiplex assay (Luminex<sup>®</sup> R&D Systems Abingdon OX, UK) according to the manufacturer's instructions.

25OH D<sub>3</sub> in serum was analysed by chemiluminescence immunoassay (CLIA) on a LIAISON instrument (DiaSorin Inc., Stillwater, MN, USA) detectable range 7.5–175 nmol/l, CV 2–5%.

Faecal calprotectin was analysed by ELISA (Buhlmann labs, Schönenbuch, Switzerland). Cholesterol and HDL were analysed by an enzymatic assay adapted to photometric analysis. High-sensitivity CRP levels (hsCRP), Apo A1 and ApoB were analysed by immunoturbidimetric assays. These analyses were performed on serum and plasma samples, respectively, at the Department of Clinical Chemistry, Karolinska University Hospital according to standardized and accredited methods.

*Statistical analysis.* For unadjusted comparisons, Mann–Whitney  $U$ -test was used for continuous variables and the Fisher exact test for dichotomous variables. To allow robust statistical analysis, IFN- $\gamma$ , IL-4, IL-10 and IL-17 were dichotomized due to undetectable levels in a large fraction of both HIV patients and controls. Faecal calprotectin-levels were subjected to dichotomy for similar reasons.

Differences in vitamin D levels between HIV patients and controls were investigated using a linear regression model fitted with possible confounders (age, BMI and smoking).

Associations between inflammatory markers and vitamin D in HIV patients and controls were adjusted for the same confounders as above (age, BMI and smoking). In this analysis, hsCRP was dichotomized with a cut point of one due to undetectable levels in six samples (2 HIV and 4 controls), and a skewed profile with extreme outliers in the HIV population. Linear regression was used when the inflammatory markers were treated as continuous (sCD14, LPS, MCP-1). Logistic regression analysis was conducted when the inflammatory markers were treated as binary (hsCRP, f-calprotectin, IFN- $\gamma$ , IL-4, IL-10 and IL-17). For IL-6, we used ordinal logistic regression to compensate for a skewed distribution.

In order to analyse possible explanations to the difference in sCD14 and hsCRP seen in HIV patients

and controls, linear and logistic regression were used, respectively, controlling for age, smoking and cholesterol levels as possible confounders.

## Results

### Baseline characteristics

The HIV cohort had a median duration of 13 years since HIV diagnosis, current CD4<sup>+</sup> count of  $658 \times 10^6/\text{ml}$  and excellent viral control with immeasurable virus levels (<20 copies/ml) since 6.5 years (Table 1). The median CD4<sup>+</sup> nadir recorded was  $217 \times 10^6/\text{ml}$  with a maximal viral RNA load of  $260 \times 10^3$  copies/ml (Table 1). The majority (54%) had a combination treatment of nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleotide reverse transcriptase inhibitors (NNRTI). Twenty-two per cent were treated with a combination of protease inhibitors (PIs) and NRTI or NNRTI. Forty-two patients (43%) medicated with the NNRTI Efavirenz.

Both HIV patients and controls had an equal distribution of BMI (Table 2). The HIV patients were older (51 versus 42 years) and smoked more (30 versus 2%) (Table 2). In the HIV group, 25% took vitamin D (25OH D<sub>3</sub>) supplements with an equal distribution of 400–1400 IU/day and 11% were on statins (Table 2).

### Plasma levels of vitamin D in HIV patients and controls

By univariate analysis, vitamin D levels were similar with equal distribution in the two groups (Table 2). Subgroup analysis excluding the vitamin D supplemented HIV patients did not affect overall vitamin D levels, or any of the other parameters in the HIV cohort, including inflammatory or lipid biomarkers (data not shown). To

control for difference in age, BMI and smoking, which are factors known to affect vitamin D levels, a linear regression analysis was conducted. The point estimate of difference between HIV patients and controls was  $-5.1$  (95% CI  $-15.2$  to  $5.1$ ). Hence, vitamin D levels were similar in both HIV patients and controls.

The majority of both HIV patients and controls had vitamin D levels in the insufficient range, defined as 25–75 nmol/l (74% versus 77%) and both groups had an equal representation of vitamin D deficiency, defined as <25 nmol/l, (9% versus 7%). Notably, there was a seasonal shift in vitamin D levels with marginally higher values during the summer months (Fig. 1) Although HIV patients and controls were recruited unevenly over the

Table 2 Characteristics of study populations.

	HIV patients <i>n</i> = 97	Controls <i>n</i> = 30	<i>P</i> -value
Male gender, <i>n</i> (%)	97 (100)	30 (100)	
Age (years), med (IQR)	51 (46–55)	42 (36–53)	0.001
BMI, med (IQR)	25 (23–27)	25 (23–28)	0.893
Smokers, <i>n</i> (%)	30 (31)	2 (7)	0.007
Statins, <i>n</i> (%)	11 (11)	0 (0)	0.066
Vitamin D (25OH D <sub>3</sub> ) substitution, <i>n</i> (%)	24 (25)	0 (0)	0.001
Vitamin D (25OH D <sub>3</sub> ) nmol/ml, med (IQR)	50 (35–50)	56 (42–68)	0.543
hsCRP mg/ml, med (IQR)	1.40 (0.68–3.35)	0.70 (0.41–1.10)	<0.001
sCD14 µg/ml, med (IQR)	1.67 (1.47–2.08)	1.40 (1.28–1.55)	<0.001
LPS pg/ml, med (IQR)	153 (153–161)	152 (148–161)	0.498
Faecal Calprotectin <sup>a</sup> , <i>n</i> (%)	26 (27)	20 (67)	<0.001
IL-4 pos <sup>a</sup> , <i>n</i> (%)	13 (13)	2 (7)	0.518
IL-6 pg/ml, med (IQR)	1.43 (1.33–2.70)	2.7 (1.43–3.00)	<0.001
IL-10 pos <sup>a</sup> , <i>n</i> (%)	46 (47)	26 (87)	<0.001
IL-17 pos <sup>a</sup> , <i>n</i> (%)	40 (41)	25 (83)	<0.001
MCP-1 pg/ml, med (IQR)	203 (147–251)	188 (156–216)	0.435
IFN-γ pos <sup>a</sup> , <i>n</i> (%)	24 (33)	12 (40)	0.112
Tot cholesterol mmol/ml, med (IQR)	5.4 (4.65–6.30)	5.00 (4.33–5.33)	0.016
HDL cholesterol mmol/ml, med (IQR)	1.20 (1.00–3.00)	1.20 (0.98–1.40)	0.582
Non-HDL cholesterol mmol/ml, med (IQR)	4.10 (3.50–5.05)	3.80 (3.10–3.30)	0.010
Apo A1 mg/ml, med (IQR)	1.42 (1.24–1.57)	1.34 (1.24–1.52)	0.514
Apo B mg/ml, med (IQR)	1.00 (0.86–1.20)	0.94 (0.76–1.06)	0.009
Apo B/Apo A1, med (IQR)	0.74 (0.62–0.86)	0.65 (0.55–0.76)	0.018

N (%): number (percentage), med (IQR): median (interquartile range).

<sup>a</sup>Due to undetectable values in a large fraction of patients, faecal calprotectin, IL-4, IL-10, IL-17 and IFN-γ, were dichotomized into positive/negative. Mann-Whitney *U*-test was used for continuous variables and Fisher exact test for dichotomous variables.

Table 1 HIV patient's characteristics.

HIV patients <i>n</i> = 97	
Years since HIV diagnosis, <i>n</i> (IQR)	13 (7–21)
CD 4 nadir, cells/µl (IQR)	217 (129–276)
Max viral load, $\times 10^3$ copies/ml (IQR)	260 (83.3–565.5)
Present CD 4, cells/µl (IQR)	658 (519–873)
Present viral load, copies/ml, (%)	<20 (100)
Years with viral load <20 copies/ml (IQR)	6.5 (3.5–9.5)
HAART, <i>n</i> (%):	97 (100)
2 NRTI + PI	20 (21)
NRTI + NNRTI + PI	1 (1)
NRTI + NNRTI	52 (54)
II + PI	4 (3)
NRTI + II	13 (13)
Other	7 (7)

Median values if not otherwise stated. HAART, highly active anti-retroviral therapy; NRTI, nucleotide reverse transcriptase inhibitors; NNRTI, non-nucleotide reverse transcriptase inhibitors; PI, protease inhibitors; II, integrase inhibitors.

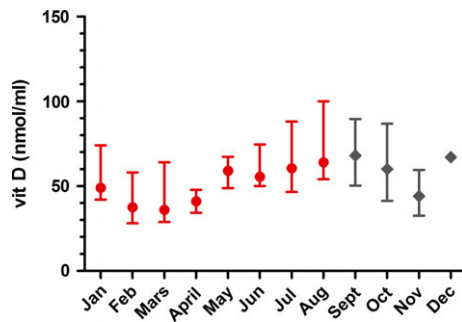


Figure 1 25OH D<sub>3</sub> levels over time. Median and IQR levels of vitamin D. Red circles represent HIV patients, and black squares represent controls.

year (HIV patients February–August, controls September–December), this did not significantly affect the group median values (50 nmol/l [35–50] versus 56 nmol/l [42–68],  $P = 0.543$ ). However, when excluding the months of May–August, when the solar intensity is strong enough to stimulate vitamin D synthesis in Stockholm (58 degrees north), the median levels of vitamin D were significantly lower in the remaining HIV patients ( $n = 53$ ) compared to controls (42 nmol/l [31–63] versus 56 nmol/l [41–68],  $P = 0.044$ ). Furthermore, in the HIV group, median levels of vitamin D were significantly lower among smokers than non-smokers (41 nmol/l [44–70] versus 57 nmol/l [44–70],  $P = 0.037$ ).

#### Microbial translocation and inflammation

Despite long-standing viral control, the HIV patients in this cohort displayed a significantly higher degree of systemic inflammatory activity with increased levels of sCD14 and hsCRP (Table 2). However, contrary to other studies, markers of microbial translocation and immune activation in the form of LPS, MCP-1 and IFN- $\gamma$  levels were not significantly different between the two groups. Furthermore, analyses of dichotomized f-calprotectin and the cytokines IL-17 and IL-10, demonstrated a higher prevalence in the controls. Unexpectedly, IL-6 levels were higher in the controls, (Table 2). No correlation between sCD14 and hsCRP, or any of the other inflammatory markers could be detected (data not shown).

#### Vitamin D levels and association with microbial translocation and inflammation

To address the question, whether vitamin D levels were associated with markers of MT and systemic immune activation, regression analyses were conducted for sCD14, hsCRP, LPS, f-calprotectin, MCP-1, IFN- $\gamma$ , IL-4, IL-6, IL-10 and IL-17. When controlling for age, BMI, vitamin D substitution and smoking, none of the estimates were statistically significant (Table 3), suggesting that vitamin

Table 3 Estimates of interaction between 25OH D<sub>3</sub> and microbial translocation and markers of systemic inflammation in HIV and control.

Dependent variable	Beta-coefficient	95% CI	P-value
sCD14	0.00	−0.00, 0.01	0.140
LPS	0.01	−0.39, 0.42	0.940
MCP-1	−0.11	−1.05, 0.83	0.820
Calprotectin	0.00	−0.05, 0.04	0.860
IFN $\gamma$	−0.03	−0.08, 0.01	0.140
IL4	0.02	−0.06, 0.10	0.670
IL6	−0.02	−0.07, 0.02	0.330
IL10	−0.01	−0.06, 0.05	0.840
IL17	−0.04	−0.09, 0.02	0.160
hsCRP	0.01	−0.04, 0.06	0.640

Multiple regression analysis of vitamin D, microbial translocation and systemic inflammation, adjusting for age, BMI, vitamin D supplementation and smoking. Linear regression was used for continuous markers (sCD14, LPS, MCP-1). Logistic regression analysis was conducted when the inflammatory markers were treated as binary (hsCRP, f-calprotectin, IFN- $\gamma$ , IL-4, IL-10 and IL-17). HsCRP was dichotomized with a cut point of 1 due to undetectable levels in 6 samples (2 HIV and 4 controls), and a skewed profile in the HIV population. Logistic regression was also used for IL-6 to compensate for a skewed distribution.

D levels were not associated with inflammatory markers in either group.

A subgroup analysis was performed on vitamin D-deficient HIV patients (<25 nmol/l;  $n = 9$ , (9%)). They were younger (49 years [45–51] versus 51 [46–56],  $P = 0.019$ ) than patients with vitamin D levels >25 nmol/l and none were on vitamin D supplements, but did not differ in other baseline characteristics. The vitamin D-deficient HIV patients had significantly higher IL-6 levels (2.7 pg/ml [2.7–4.8] versus 1.3 [1.3–2.7],  $P = 0.003$ ). There was, however, no significant difference in levels of hsCRP, sCD14, LPS, f-calprotectin, IL-4, IL-10, IL-17, MCP-1 or IFN- $\gamma$ . Nor was there any association between vitamin D levels and markers of MT and inflammation (data not shown).

#### Lipid status and association with microbial translocation, inflammation and vitamin D

Levels of total cholesterol, non-HDL cholesterol, Apo B and ApoB/ApoA1 were significantly higher in HIV patients compared to controls (Table 2). LPS associated with total cholesterol in HIV patients and controls ( $r = 0.29$ ,  $P = 0.004$  versus  $r = 0.38$ ,  $P = 0.033$ ), and in HIV patients also with Apo B ( $r = 0.21$ ,  $P = 0.029$ ) and non-HDL cholesterol ( $r = 0.35$ ,  $P = 0.001$ ). However, no association was found between lipids and sCD14, hsCRP or any of the other inflammatory markers in either group (data not shown).

Moreover, no association was found between plasma lipids and vitamin D levels in HIV patients and controls, not even in the vitamin D-deficient HIV subgroup (data not shown).

## HIV is an independent risk factor for increased sCD14 and hsCRP

To investigate why our well-treated HIV patients had higher levels of sCD14 and hsCRP, two regression analyses per inflammatory marker were conducted; one with HIV as an independent variable and one where possible confounders were added. We chose age, smoking and cholesterol levels as these are variables known to affect inflammation, but also because these variables were significantly higher in the HIV cohort. The independent variables neither changed the estimates for HIV, nor changed the confidence interval substantially (Table 4). Thus, HIV infection remained an independent risk factor for the increased levels of sCD14 and hsCRP present in the HIV cohort.

## Discussion

### Statement of principal findings

The main finding of this study is that vitamin D levels did not differ between chronic HIV patients and controls. Vitamin D levels were similarly distributed and equally low in both groups. There was no discernable association between vitamin D levels in serum and markers of MT, systemic inflammation or dyslipidemia. Levels of LPS were not increased in the HIV cohort and f-calprotectin did not serve as a valuable marker to evaluate the degree of intestinal disturbance or inflammation. However, despite perfect virological control for many years, these HIV patients still expressed a higher degree of immune

activation, as detected by increased levels of the monocyte activation marker sCD14 and the acute-phase-reactant hsCRP, with HIV infection as an independent risk factor.

### Strengths and weaknesses

To our recollection, this is the first study of vitamin D in relation to MT, inflammatory activation and blood lipids in a uniquely homogenous cohort of Swedish HIV patients of Caucasian descent on HAART with long-standing viral control (immeasurable viral load and CD4 levels within normal range since median 6.5 years). The cohort has been followed for several years and is well documented in terms of metabolic and cardiovascular risk factors as well as HIV viral control and medical adherence. They have no other known inflammatory diseases, or co-infections and share the same genetic background as the majority of the Swedish population.

We believe that this group is representative of a majority of Caucasian HIV patients in Sweden and might also represent a large portion of chronic HIV patients in the western world. Furthermore, this study it is one of the first where faecal calprotectin is analysed in chronic HIV patients as a marker of intestinal inflammation.

We acknowledge that the analyses might be affected by limited sample size and unmatched controls in terms of age. One-fourth of the HIV populations took vitamin D supplements ranging from 400 to 1400 IU/day (400 IU:  $n = 8$ , 800 IU:  $n = 11$ , 1400 IU:  $n = 8$ ) with a possible confounding effect. However, if vitamin D supplementation at this level indeed influenced MT, inflammatory and lipid biomarkers, it could be expected that those individuals would differ from the non-supplemented patients with regard to the analysed parameters. Importantly, we could not observe such an effect. However, we cannot rule out that those patients on vitamin D supplementation had disturbed markers before the supplementation was initiated. It should be noted that the study was not designed to study the interventional role of vitamin D but rather constitutes a cross-sectional study to prepare for a future interventional trial.

A major limitation of the study is that the HIV and control samples were taken at different time points over the year when comparing vitamin D levels. HIV samples were collected from January to August, whereas controls were collected from September to December. Indeed, analyses of vitamin D levels excluding the summer months (May–August) when the solar intensity is strong enough to stimulate vitamin D synthesis [37], demonstrated significantly lower vitamin D levels in the HIV cohort ( $P = 0.044$ ). However, a subgroup analysis of vitamin D-deficient HIV patients showed that, other than IL-6, there was no difference in levels of microbial translocation, systemic inflammation or dyslipidemia. Nor was there a correlation between vitamin D levels, LPS, inflammatory

**Table 4** Association between HIV and hsCRP, or sCD14 in HIV patients.

Inflammation marker	Independent variable	Estimate	95% CI	P-value
hsCRP (model 1)	HIV	1.50	0.59, 2.40	0.001
hsCRP (model 2)	HIV	1.46	0.42, 2.50	0.006
	Vitamin D	−0.00	−0.02, 0.01	0.704
	Age	0.00	−0.05, 0.05	0.915
	BMI	0.17	0.03, 0.30	0.017
	Smoker	0.47	−0.46, 1.41	0.321
sCD14 (model 1)	HIV	0.368	0.242, 0.493	<0.001
sCD14 (model 2)	HIV	0.394	0.247, 0.540	<0.001
	Vitamin D	0.001	−0.003, 0.005	0.573
	Age	−0.004	−0.012, 0.004	0.375
	BMI	−0.009	−0.031, 0.013	0.431
	Smoker	0.008	−0.196, 0.212	0.940

Regression analysis of the inflammation markers hsCRP and sCD14 in the HIV cohort, with and without the variables vitamin D, age, BMI and smoking, demonstrating that HIV is an independent risk factor behind the observed inflammation. HsCRP was treated as binary with a cut point of one due to undetectable levels in 2 samples and a skewed distribution and analyzed by logistic regression with the estimate representing log-odds ratio. Linear regression was used for sCD14 with the estimate representing beta coefficient.

markers or lipids. Moreover, a study of intra-individual variations in serum vitamin D levels in a healthy Swedish adult population with a mean age of  $40.5 \pm 13.0$  years demonstrated very similar levels and distribution over the months [38]. The incidence of vitamin D deficiency (<25 nmol/l) was also strikingly similar to those we found in the HIV cohort and controls (5.1% versus 9% and 7%, respectively). The notion that the vitamin D levels recorded in this study may represent a Swedish population living at the 58th latitude is further corroborated by a previous patient study performed by our group in Stockholm Sweden, demonstrating similar median vitamin D levels (50 nmol/l) [39] as the ones demonstrated in the HIV cohort (50 nmol/l) and controls (56 nmol/l) of this study.

### Relation to other studies

In concordance with previously published studies, this cohort of chronic HIV patients exhibited enhanced levels of sCD14 and hsCRP [40, 41], as well as a disturbed lipid profile with increased levels of 'bad' cholesterol (total cholesterol, non-HDL cholesterol and Apo B) [42]. However, we did not observe an overrepresentation of vitamin D deficiency, nor did we see an association between vitamin D deficiency and increased systemic inflammation as demonstrated by two recent European studies by Ansemant *et al.* and Legai *et al.* [43, 44].

Possible explanations to this observed disparity could be the differences in study populations. Compared with our study, their results are based on more heterogeneous HIV cohorts of mixed sex and ethnical origin with a large percentage of black participants and patients co-infected with hepatitis. They also had larger study populations (Ansemant no = 263, Legai no = 355) whereas our study is relatively small with an inherent risk for type II error, masking a true difference between the groups. It is also possible that the differences in vitamin D levels are caused by residual confounders and lifestyle factors not controlled for. In fact, multivariate analysis showed that smoking was positively associated with vitamin D deficiency in both of these studies. This result is also reflected in our study where HIV patients that smokes have significantly lower median levels of vitamin D.

### Meaning of the study

The overall aim of the current study was to provide baseline data for a future interventional trial testing the hypothesis that vitamin D could reduce microbial translocation, dampen inflammation and have beneficial effects on blood lipids. As the associations between these outcome measurements and vitamin D status appear to be weak in chronic Swedish HIV patients, the rationale for such an interventional trial is not convincing. One might argue

that the increased levels of hsCRP and sCD14 seen in the HIV cohort may correlate more to smoking and other lifestyle-associated confounders, as suggested by Valiathan *et al.* [45].

However, vitamin D supplementation could still play a role in curbing acute inflammation in the early phase of HIV disease where gut enteropathy is pronounced and levels of LPS is markedly enhanced. An interventional study on vitamin D supplementation in patients with chronic kidney disease demonstrated reduced levels of endotoxins measured by the Endotoxin Activity Assay [46]. Similarly, we cannot rule out that vitamin D could have positive effects if given very early in the HIV disease process, but this speculation needs to be tested in clinical trials. In addition, it is possible that vitamin D contributes to the protection against concomitant bacterial infections in HIV patients, as a randomized and placebo-controlled trial on vitamin D supplementation in HIV-infected youths showed an enhanced antibacterial immune response [35].

### In conclusion

We find that well-controlled HIV patients on long-term HAART have similar vitamin D levels as controls. Despite perfect virological control, the HIV-group exhibit a slightly increased inflammatory component and disturbed blood lipids. However, neither of these parameters were associated with low vitamin D levels but appeared to be exclusively linked with the HIV-disease *per se*. Thus, we do not foresee a large interventional trial where vitamin D given to chronic HIV patients in Sweden, but rather suggest that HIV patients are continuously monitored for life style factors known to influence inflammatory disease with strong recommendations to stop smoking.

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### References

- 1 Allers K, Fehr M, Conrad K *et al.* Macrophages accumulate in the gut mucosa of untreated HIV-infected patients. *J Infect Dis* 2014;209:739–48.
- 2 Deeks SG, Tracy R, Douek DC. Systemic effects of inflammation on health during chronic HIV infection. *Immunity* 2013;39:633–45.
- 3 Marchetti G, Cozzi-Lepri A, Merlini E *et al.* Microbial translocation predicts disease progression of HIV-infected antiretroviral-naïve patients with high CD4+ cell count. *AIDS (London, England)* 2011;25:1385–94.

- 4 Kuller LH, Tracy R, Bellosso W *et al.* Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med* 2008;5:e203.
- 5 Thiebaut R, Charpentier C, Damond F *et al.* Association of soluble CD14 and inflammatory biomarkers with HIV-2 disease progression. *Clin Infect Dis* 2012;55:1417–25.
- 6 Sandler NG, Wand H, Roque A *et al.* Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J Infect Dis* 2011;203:780–90.
- 7 Hunt PW, Sinclair E, Rodriguez B *et al.* Gut epithelial barrier dysfunction and innate immune activation predict mortality in treated HIV infection. *J Infect Dis* 2014;210:1228–1238.
- 8 Shepherd L, Souberbielle JC, Bastard JP *et al.* Prognostic value of vitamin D level for all-cause mortality, and association with inflammatory markers, in HIV-infected persons. *J Infect Dis* 2014;210:234–43.
- 9 Viard JP, Souberbielle JC, Kirk O *et al.* Vitamin D and clinical disease progression in HIV infection: results from the EuroSIDA study. *AIDS (London, England)*. 2011;25:1305–15.
- 10 Sudfeld CR, Wang M, Aboud S, Giovannucci EL, Mugusi FM, Fawzi WW. Vitamin D and HIV progression among Tanzanian adults initiating antiretroviral therapy. *PLoS ONE* 2012;7:e40036.
- 11 Vescini F, Cozzi-Lepri A, Borderi M *et al.* Prevalence of hypovitaminosis D and factors associated with vitamin D deficiency and morbidity among HIV-infected patients enrolled in a large Italian cohort. *J Acquir Immune Defic Syndr* 2011;58:163–72.
- 12 Havers F, Smeaton L, Gupte N *et al.* 25-Hydroxyvitamin D insufficiency and deficiency is associated with HIV disease progression and virological failure post-antiretroviral therapy initiation in diverse multinational settings. *J Infect Dis* 2014;210:244–53.
- 13 Mehta S, Mugusi FM, Spiegelman D *et al.* Vitamin D status and its association with morbidity including wasting and opportunistic illnesses in HIV-infected women in Tanzania. *AIDS Patient Care STDs* 2011;25:579–85.
- 14 Choi AI, Lo JC, Mulligan K *et al.* Association of vitamin D insufficiency with carotid intima-media thickness in HIV-infected persons. *Clin Infect Dis* 2011;52:941–4.
- 15 Lai H, Gerstenblith G, Fishman EK *et al.* Vitamin D deficiency is associated with silent coronary artery disease in cardiovascularly asymptomatic African Americans with HIV infection. *Clin Infect Dis* 2012;54:1747–55.
- 16 Ginde AA, Scragg R, Schwartz RS, Camargo CA Jr. Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. adults. *J Am Geriatr Soc* 2009;57:1595–603.
- 17 Ross AC, Judd S, Kumari M *et al.* Vitamin D is linked to carotid intima-media thickness and immune reconstitution in HIV-positive individuals. *Antivir Ther* 2011;16:555–63.
- 18 Wang L, Song Y, Manson JE *et al.* Circulating 25-hydroxy-vitamin D and risk of cardiovascular disease: a meta-analysis of prospective studies. *Circ Cardiovasc Qual Outcomes* 2012;5:819–29.
- 19 Hammond E, McKinnon E, Glendenning P, Williams R, Mallal S, Phillips E. Association between 25-OH vitamin D and insulin is independent of lipotrophy in HIV. *Clin Endocrinol* 2012;76:201–6.
- 20 Szep Z, Guaraldi G, Shah SS *et al.* Vitamin D deficiency is associated with type 2 diabetes mellitus in HIV infection. *AIDS (London, England)* 2011;25:525–9.
- 21 Stadlmayr A, Aigner E, Huber-Schonauer U *et al.* Relations of vitamin D status, gender and type 2 diabetes in middle-aged Caucasians. *Acta Diabetol* 2015;52:39–46.
- 22 Gagnon C, Lu ZX, Magliano DJ *et al.* Low serum 25-hydroxyvitamin D is associated with increased risk of the development of the metabolic syndrome at five years: results from a national, population-based prospective study (The Australian Diabetes, Obesity and Lifestyle Study: AusDiab). *J Clin Endocrinol Metab* 2012;97:1953–61.
- 23 Dao CN, Patel P, Overton ET *et al.* Low vitamin D among HIV-infected adults: prevalence of and risk factors for low vitamin D Levels in a cohort of HIV-infected adults and comparison to prevalence among adults in the US general population. *Clin Infect Dis* 2011;52:396–405.
- 24 Fox J, Peters B, Prakash M, Arribas J, Hill A, Moecklinghoff C. Improvement in vitamin D deficiency following antiretroviral regime change: results from the MONET trial. *AIDS Res Hum Retroviruses* 2011;27:29–34.
- 25 Schwartz JB, Moore KL, Yin M *et al.* Relationship of vitamin D, HIV, HIV treatment, and lipid levels in the Women's Interagency HIV Study of HIV-infected and uninfected women in the United States. *J Int Assoc Provid AIDS Care* 2014;13:250–9.
- 26 Assa A, Vong L, Pinnell LJ, Avitzur N, Johnson-Henry KC, Sherman PM. Vitamin D deficiency promotes epithelial barrier dysfunction and intestinal inflammation. *J Infect Dis* 2014;210:1296–1305.
- 27 Zhao H, Zhang H, Wu H *et al.* Protective role of 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub> in the mucosal injury and epithelial barrier disruption in DSS-induced acute colitis in mice. *BMC Gastroenterol* 2012;12:57.
- 28 Lagishetty V, Misharin AV, Liu NQ *et al.* Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. *Endocrinology* 2010;151:2423–32.
- 29 Misawa Y, Baba A, Ito S, Tanaka M, Shiohara M. Vitamin D(3) induces expression of human cathelicidin antimicrobial peptide 18 in newborns. *Int J Hematol* 2009;90:561–70.
- 30 Campbell GR, Spector SA. Vitamin D inhibits human immunodeficiency virus type 1 and Mycobacterium tuberculosis infection in macrophages through the induction of autophagy. *PLoS Pathog* 2012;8:e1002689.
- 31 Liu W, Chen Y, Golan MA *et al.* Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. *J Clin Invest* 2013;123:3983–96.
- 32 Garg M, Rosella O, Lubel JS, Gibson PR. Association of circulating vitamin D concentrations with intestinal but not systemic inflammation in inflammatory bowel disease. *Inflamm Bowel Dis* 2013;19:2634–43.
- 33 Giacomet V, Vigano A, Manfredini V *et al.* Cholecalciferol supplementation in HIV-infected youth with vitamin D insufficiency: effects on vitamin D status and T-cell phenotype: a randomized controlled trial. *HIV Clin Trials* 2013;14:51–60.
- 34 Stallings VA, Schall JI, Hediger ML *et al.* High-dose vitamin D<sub>3</sub> supplementation in children and young adults with HIV: a randomized, placebo-controlled trial. *Pediatr Infect Dis J* 2014;34:32–40.
- 35 Chun RF, Liu NQ, Lee T *et al.* Vitamin D supplementation and antibacterial immune responses in adolescents and young adults with HIV/AIDS. *J Steroid Biochem Mol Biol* 2015;148:290–297.
- 36 Longenecker CT, Hileman CO, Carman TL *et al.* Vitamin D supplementation and endothelial function in vitamin D deficient HIV-infected patients: a randomized placebo-controlled trial. *Antivir Ther* 2012;17:613–21.
- 37 Webb AR, Kline L, Holick MF. Influence of season and latitude on the cutaneous synthesis of vitamin D<sub>3</sub>: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D<sub>3</sub> synthesis in human skin. *J Clin Endocrinol Metab* 1988;67:373–8.
- 38 Klingberg E, Olerod G, Konar J, Petzold M, Hammarsten O. Seasonal variations in serum 25-hydroxy vitamin D levels in a Swedish cohort. *Endocrine* 2015; [Epub ahead of print].
- 39 Bergman P, Norlin AC, Hansen S *et al.* Vitamin D<sub>3</sub> supplementation in patients with frequent respiratory tract infections: a randomised and double-blind intervention study. *BMJ Open* 2012;2:1–10.
- 40 Wilson EM, Singh A, Hullsiek KH *et al.* Monocyte-activation phenotypes are associated with biomarkers of inflammation and

- coagulation in chronic HIV infection. *J Infect Dis* 2014; 210:1396–1406.
- 41 Armah KA, McGinnis K, Baker J *et al.* HIV status, burden of comorbid disease, and biomarkers of inflammation, altered coagulation, and monocyte activation. *Clin Infect Dis* 2012;55:126–36.
- 42 Pedersen KK, Pedersen M, Troseid M *et al.* Microbial translocation in HIV infection is associated with dyslipidemia, insulin resistance, and risk of myocardial infarction. *J Acquir Immune Defic Syndr* 2013;64:425–33.
- 43 Ansemant T, Mahy S, Piroth C *et al.* Severe hypovitaminosis D correlates with increased inflammatory markers in HIV infected patients. *BMC Infect Dis* 2013;13:7.
- 44 Legeai C, Vigouroux C, Souberbielle JC *et al.* Associations between 25-hydroxyvitamin D and immunologic, metabolic, inflammatory markers in treatment-naive HIV-infected persons: the ANRS CO9 <<COPANA>> cohort study. *PLoS ONE* 2013;8:e74868.
- 45 Valiathan R, Miguez MJ, Patel B, Arheart KL, Asthana D. Tobacco smoking increases immune activation and impairs T-cell function in HIV infected patients on antiretrovirals: a cross-sectional pilot study. *PLoS ONE* 2014;9:e97698.
- 46 Ponda MP, Breslow JL. Vitamin D<sub>3</sub> Repletion in Chronic Kidney Disease Stage 3: effects on Blood Endotoxin Activity, Inflammatory Cytokines, and Intestinal Permeability. *Ren Fail* 2013;35:497–503.