Corticosteroid Therapy, Vitamin D Status, and Inflammatory Cytokine Profile in the HIV-Tuberculosis Immune Reconstitution Inflammatory Syndrome

Anali Conesa-Botella,[1,2,3] Graeme Meintjes,[1,4,5,6] Anna K. Coussens,[7] Helen van der Plas,[6] Rene Goliath,[1] Charlotte Schutz,[1,5] Rodrigo Moreno-Reyes,[8] Meera Mehta,[3] Adrian R. Martineau,[4,7,9] Robert J. Wilkinson,[1,4,5,6,7] Robert Colebunders,[2,3] and Katalin A. Wilkinson[1,6,7]

[1]Clinical Infectious Diseases Research Initiative, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory, South Africa; [2]Department of Clinical Sciences, Institute of Tropical Medicine, [3]Epidemiology and Social Medicine, University of Antwerp, Belgium; [4]Division of Medicine, Imperial College London, United Kingdom; [5]Infectious Diseases Unit, GF Jooste Hospital, Manenberg, [6]Department of Medicine, University of Cape Town, Observatory, South Africa; [7]Division of Mycobacterial Research, MRC National Institute for Medical Research, London, United Kingdom; [8]Department of Nuclear Medicine, Erasme Hospital, Free University of Brussels, Belgium; and [9]Centre for Primary Care and Public Health, Queen Mary University of London, United Kingdom

Background. Tuberculosis-immune reconstitution inflammatory syndrome (TB-IRIS) in patients coinfected with human immunodeficiency virus (HIV) and tuberculosis starting antiretroviral therapy (ART) is associated with hypercytokinemia. As adjunctive corticosteroid therapy and vitamin D have immunomodulatory properties, we investigated the relationship between cytokine/chemokine profiles, corticosteroid use, and vitamin D deficiency in TB-IRIS patients.

Methods. Plasma from 39 TB-IRIS and 42 non-IRIS patients was collected during a prospective study of HIV-associated tuberculosis patients starting ART. In total, 26% of patients received corticosteroid (CTC) therapy pre-ART for severe tuberculosis. Concentrations of total 25-hydroxyvitamin D (25(OH)D) and 14 cytokines/chemokines were determined at ART initiation and 2 weeks later.

Results. Patients prescribed concurrent CTC had lower interferon γ (IFN-γ), IP-10, tumor necrosis factor (TNF), interleukin (IL)-6, IL-8, IL-10, IL-12p40, and IL-18 pre-ART ($P \leq .02$). TB-IRIS presented at 12 days (median) of ART, irrespective of CTC use. In patients who developed TB-IRIS (not on CTC) IL-6, IL-8, IL-12p40, IL-18, IP-10, and TNF increased during 2 weeks ($P \leq .04$) of ART. Vitamin D deficiency (total 25(OH)D <75 nmol/L) was highly prevalent (89%) at baseline. Although vitamin D deficiency at either baseline or 2 weeks was not associated with TB-IRIS, in those not on CTC the median 25(OH)D decreased during 2 weeks ($P = .004$) of ART. Severe vitamin D deficiency (total 25(OH)D <25 nmol/L) was associated with higher baseline TNF, IL-6, and IL-8 irrespective of IRIS status.

Conclusions. CTC modifies the inflammatory profile of those who develop TB-IRIS. The association between severe vitamin D deficiency and elevated proinflammatory cytokines support a study of vitamin D supplementation in HIV-TB co-infected patients starting ART.

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Correspondence: Katalin A. Wilkinson, PhD, Institute of Infectious Disease and Molecular Medicine, Clinical and Infectious Disease Research Initiative, Observatory 7925, Cape Town, South Africa (Katalin.Wilkinson@uct.ac.za).

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Africa accounts for 82% of the global human immunodeficiency virus type 1 (HIV-1) associated tuberculosis burden [1]. Combined antiretroviral therapy (ART) rollout has grown steadily, with 54% of HIV-infected tuberculosis patients started on ART in South Africa [1]. When HIV-1-infected persons start ART, the resulting rise in CD4 count and improved immune function partially restore pathogen specific immunity [2]. However, 8%–43% of patients will experience paradoxical

deterioration, termed tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) [3, 4]. Initiating ART at lower CD4 count and advanced tuberculosis disease are the main factors associated with this syndrome [4]. We have previously shown an association between increased circulating proinflammatory cytokines (tumor necrosis factor [TNF], interleukin [IL]-6, and interferon γ [IFN-γ]) and TB-IRIS [5]. Corticosteroids (CTC) are antiinflammatory molecules, which have an inhibitory effect on proinflammatory T-cells while stimulating antiinflammatory and regulatory T cells [6]. Our randomized placebo-controlled trial of prednisone for the treatment of paradoxical TB-IRIS showed reduced duration of hospitalization and therapeutic procedure numbers, as well as hastened improvements in TB-IRIS symptoms, and more rapid reduction in C-reactive protein (CRP) [7].

We also recently showed that vitamin D deficiency is highly prevalent in Cape Town and is associated with active tuberculosis in both HIV-uninfected and infected patients, in whom the association is stronger [8]. Others have shown that patients with tuberculosis have significantly lower 25-hydroxyvitamin D (25(OH)D) levels than those without in Tanzania and West Africa [9, 10]. These findings are supported by the requirement of 25(OH)D for activating an IFN-γ mediated antimicrobial effector pathway via induction of antimicrobial peptide synthesis and autophagy in human monocytes and macrophages [11], highlighting the importance of adequate amounts of 25(OH)D for sustaining innate and acquired immunity against infections. In vitro, 1,25-dihydroxyvitamin D has also been shown to inhibit leukocyte secretion of IL-6, IL-12p40, and IFN-γ, while inducing IL-10 [12–14] and regulatory T-cell polarisation [15], suggesting that one of its primary roles is to prevent chronic inflammation and limit immunopathology.

As TB-IRIS has been associated with hypercytokinemia and both HIV and tuberculosis treatment are known to interfere with vitamin D metabolism [16, 17], we hypothesised that vitamin D deficiency may be associated with TB-IRIS development. We therefore assessed the prevalence of vitamin D deficiency in a cohort of HIV-1 and tuberculosis coinfected patients in Cape Town and investigated the relationship between 25(OH)D and plasma cytokines/chemokines and TB-IRIS development, in patients stratified by CTC use.

MATERIALS AND METHODS

Study Population

HIV-1-tuberculosis coinfected patients admitted to Brooklyn Chest Hospital (Cape Town, South Africa) for severe tuberculosis, started ART while on tuberculosis treatment in the ward between June 2009 and December 2010. All were inpatients and monitored closely for 12 weeks after starting ART for development of TB-IRIS and gave written informed consent for inclusion in the study. Patients with available plasma at both baseline and week 2 of ART (81 of 105 enrolled) were included in this study. Our sample was representative of the entire cohort. The University of Cape Town Research Ethics Committee approved the study (REC 049/2009).

Definitions

Tuberculosis diagnosis was based on smear or culture positivity. Where this was negative or unavailable, diagnosis according to World Health Organization (WHO) guidelines for smear-negative or extrapulmonary tuberculosis in HIV-1-infected persons [18]. TB-IRIS was diagnosed according to a published case definition [4]. "Non-IRIS" patients were IRIS-free during the 3-month follow-up period. Severe vitamin D deficiency was defined as total 25(OH)D ≤25 nmol/L, moderate as 25(OH)D between 25 and 50 nmol/L, and suboptimal as 25(OH)D between 50 and 75 nmol/L [19]. Severe TB-IRIS was defined by the presence of at least 4/8 symptoms from the following categories: neurological, pulmonary, abdominal, increase or occurrence of new lymph nodes, presence of effusion (pulmonary, cardiac, or ascites), occurrence of fever, a heart rate above 120, and level of CRP above the median of 93 mg/L.

Sampling and Laboratory Assays

Plasma was obtained at the start of ART and 2 weeks postinitiation and stored at −80°C for batched analysis. Total plasma 25(OH)D (25(OH)D_2 and 25(OH)D_3) was measured by radioimmunoassay (DiaSorin) in duplicate. Quality control was performed by the vitamin D External Quality Assurance Survey (DEQAS, www.deqas.org). To assess the association between vitamin D status and hypercytokinemia, 14 cytokines/chemokines were measured based on our previous study [5], in 39 IRIS and 38 non-IRIS patients with available sample. Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-α2, Interferon gamma-induced protein 10 (IP-10), macrophage inflammatory protein (MIP)-1α, MIP-1β, TNF, IFN-γ, IL-2, IL-6, IL-8, IL-10, IL-12p40, and IL-17 were quantified on the Bio-Plex platform (Bio-Rad Laboratories, Hercules, United States), using customized Milliplex kits (HCYTMAG-60K, Millipore, Missouri) and IL-18 by enzyme-linked immunosorbent assay (ELISA; Medical&Biological Laboratories, Japan). Cytokine concentrations below the limit of detection (LOD) were considered zero.

Statistical Analysis

Statistical analyses were performed using Stata software (version 10.2; StataCorp, Texas) and GraphPad Prism software (version 5; GraphPad, San Diego, California). Baseline characteristics, 25(OH)D and cytokine levels were summarized by count and proportion (%) or median with interquartile

range (IQR). Normality was assessed using graphical procedures. All P values reported were 2-sided at α of 0.05. Differences between patient groups were assessed by Fisher exact test for proportions, and Mann-Whitney U test for medians. Differences in cytokine and 25(OH)D concentrations were compared using Mann-Whitney U test or Wilcoxon signed rank test for paired data.

RESULTS

Patient Characteristics

In total, 39 of 81 patients developed IRIS during the longitudinal follow-up at 12 days median (IQR: 7–19) into ART. All patients were on nonnucleoside reverse transcriptase inhibitor (NNRTI)-based regimen: an NRTI backbone and either Efavirenz (n = 79) or Nevirapine (n = 2). Eight persons in the IRIS group and 13 in the non-IRIS group were prescribed adjunctive corticosteroid therapy (CTC) in addition to tuberculosis treatment before starting ART (Flow chart, Supplementary Figure 1). No difference was observed between the IRIS and non-IRIS groups with regard to baseline study variables (Table 1). Baseline 25(OH)D concentrations were not different among the 4 seasons.

Effect of Corticosteroids on Baseline Characteristics

CTC were prescribed to tuberculosis patients with life-threatening symptoms (thus severe tuberculosis) for median 33 days (IQR: 25–38) in TB-IRIS and 41 days (IQR: 26–50) in non-IRIS ($P = .298$) before starting ART. The numbers of TB-IRIS cases in both groups were comparable (CTC: 38.1%, non-CTC: 51.7%; $P = .287$). Both groups were also clinically similar, with the only baseline characteristic significantly different being the method of tuberculosis diagnosis: in the CTC group, more individuals were diagnosed by radiological identification (67%) rather than sputum culture (28%) or sputum smear (5%), compared with the non-CTC group, where diagnosis was predominantly by culture (51%) rather than radiological identification (37%) or sputum smear (12%), $P = .020$. Patients who received CTC pre-ART had significantly lower baseline plasma concentrations of IL-6, IL-8, IL-10, IL-12p40, IL-18, IFN-γ, IP-10, and TNF ($P \leq .016$), compared with the non-CTC group (Table 2). Because of this significant effect of CTC on the cytokine profile of patients, we stratified the data by CTC administration in further analyses.

Clinical Manifestation of IRIS in CTC and Non-CTC Groups

Although our study was not designed to analyse the differences in clinical outcome between the CTC and non-CTC groups, we noted a trend for patients not on CTC to present with more severe TB-IRIS clinical presentation, compared with patients on CTC (10/31, 32% vs 0/8, 0%); $P = .082$). Non-CTC TB-IRIS patients also showed a trend for shorter hospitalization time after starting ART, compared with those who received CTC: median, 92 days (IQR, 77–119) vs 130 days (IQR, 107–146), $P = .078$.

Table 1. Baseline Characteristics

Variable[a]	IRIS (n = 39)	Non-IRIS (n = 42)	P [b]
Male sex	16 (41)	17 (40)	.960
Age, years	33.7 (28–42)	33.5 (27–43)	.587
Time of sampling			.952
January–March	4 (10)	4 (10)	
April–June	9 (23)	9 (21)	
July–September	17 (44)	20 (48)	
September–December	9 (23)	9 (21)	
TB treatment to ART[c] (days)	36 (27–61)	34 (23–56)	.809
Viral load (copies × 10^4/mL)	67 (20.3–130)	47 (14.5–160)	.865
CD4 baseline (cells/mm^3)	80 (31–132)	67 (33.5–118)	.508
TB diagnosis			.942
Radiological	17 (43)	19 (45)	
Sputum smear	3 (8)	5 (12)	
Cultured AFB/MTB	19 (49)	18 (43)	
Type TB			.207
PTB	5 (13)	7 (17)	
EPTB	6 (15)	11 (26)	
PTB + EPTB	28 (72)	24 (57)	
WHO stage			.584
3	4 (10)	6 (14)	
4	35 (90)	36 (86)	
Pre-ART hospitalization,[d] days	18 (12–26)	14 (11–21)	.153
Corticosteroids pre-ART	9 (23)	13 (31)	.429
Time on corticosteroids, days	33 (25–38)	41 (26–50)	.298

Abbreviations: AFB, acid fast bacilli; ART, antiretroviral therapy; EPTB, extrapulmonary tuberculosis; IRIS, immune reconstitution inflammatory syndrome; MTB, *Mycobacterium tuberculosis*; PTB, pulmonary tuberculosis; TB, tuberculosis; WHO, World Health Organization,

[a] Data are No. (%) or median interquartile range.

[b] P values determined by Fisher exact test for proportions, and Mann–Whitney for medians.

[c] Time between the start of TB treatment and the start of ART.

[d] Time of hospitalization before starting ART.

Plasma Cytokines in TB-IRIS and Non-IRIS

We previously demonstrated an association between increased cytokine concentrations and TB-IRIS in a cross-sectional study at week 2 post-ART [5]. We therefore wished to establish whether differences also existed during ART and determined the plasma concentration of 14 cytokines/chemokines before and at 2 weeks of ART.

Table 2. Baseline Plasma Cytokine (pg/mL) and 25(OH)D (nmol/L) Concentrations in Patients Who Were on Corticosteroid Treatment or not on Corticosteroids Before Starting ART

Variable[a]	CTC (n = 21)	Non-CTC (n = 56)	*P* [b]
25(OH)D[c]	25.5 (19.0–38.0)	28.2 (21.1–38.7)	.360
IFN-α2	47.9 (20.4–74.5)	45.9 (16.8–96.6)	.894
IFN-γ	5.8 (1.6–15.2)	19.0 (5.9–45.3)	.004
IP-10	3194 (2105–5577)	6328 (4078–11 156)	<.001
MIP-1α	0 (0–20.2)	15.7 (0–33.8)	.124
MIP-1β	29.7 (19.9–44.2)	32.6 (25.8–55.2)	.197
TNF	22.8 (15.2–36.6)	43.7 (30.0–61.5)	<.001
IL-6	0 (0–3.6)	5.5 (0–13.8)	.005
IL-8	13.8 (8.4–20.5)	22.7 (17.0–45.7)	.001
IL-10	9.0 (4.1–15.3)	19.9 (9.0–29.6)	.016
IL-12p40	0 (0–3.9)	15.5 (0–37.3)	.002
IL-18	1129 (853–1376)	1893 (1447–2834)	.003

Abbreviations: CTC, corticosteroid; IFN, interferon; IL, interleukin; IP, inflammatory protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

[a] Data are median interquartile range.

[b] *P* values determined by Mann-Whitney test.

[c] *n* for 25(OH)D: CTC, n = 21; non-CTC, n = 60.

GM-CSF, IL-2, and IL-17 had median concentrations below the limit of detection and were not included in statistical analyses. Patients not on CTC who developed TB-IRIS showed higher baseline IFN-γ ($P = .050$), whereas those who received CTC and developed TB-IRIS showed higher IL-8 concentrations ($P = .037$) and a trend toward lower baseline IFN-α2 ($P = .054$; Figure 1 and Supplementary Table 1). At week 2 of ART, in the non-CTC group, TNF ($P = .003$), IFN-γ ($P = .004$), IL-6 ($P = .008$), and IL-8 ($P = .014$) were significantly higher in TB-IRIS patients than non-IRIS. In patients who received CTC, there were no significant differences between TB-IRIS and non-IRIS (Figure 1).

During the first 2 weeks of ART, TB-IRIS patients not on CTC showed a significant increase in IL-6, IL-8, IL-12p40, IL-18, IP-10, MIP-1β, and TNF ($P \leq .036$), whereas non-IRIS patients showed increased MIP-1α ($P = .023$) and MIP-1β ($P < .001$). Conversely, patients on CTC who developed TB-IRIS showed no significant cytokine increase during the 2 weeks of ART, whereas non-IRIS patients showed significantly increased MIP-1β ($P = .03$) and decreased IL-18 ($P = .050$, Figure 1, Supplementary Table 1). Comparing IRIS patients on CTC with those not on CTC, the increase in TNF, IL-8,

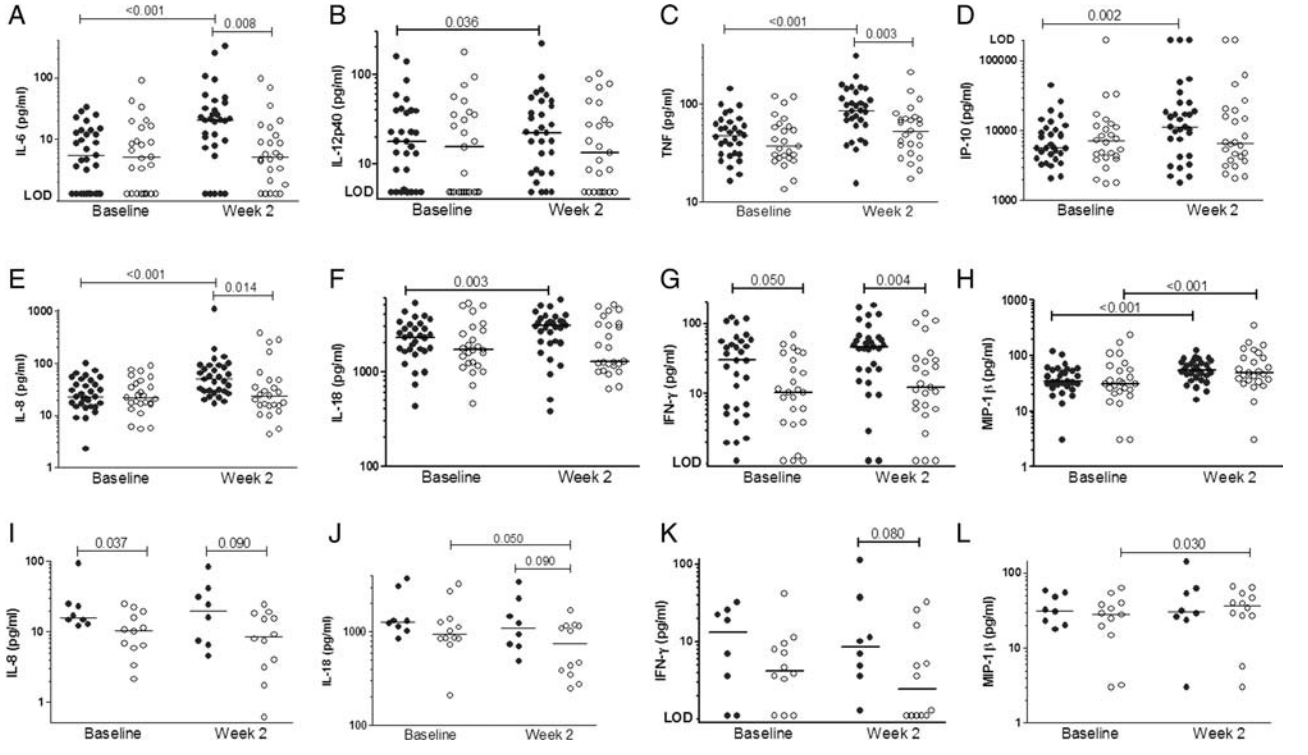

Figure 1. Cytokine concentrations in immune reconstitution inflammatory syndrome (IRIS; filled circles) and non-IRIS (open circles) patients: *A–H*, not on CTC ($n_{IRIS} = 31$, $n_{nonIRIS} = 26$); *I–L*, on CTC therapy ($n_{IRIS} = 8$, $n_{nonIRIS} = 12$). Line at median. Statistical test: Mann-Whitney (not paired data) and Wilcoxon signed rank test (paired data); significant *P* values are indicated on the graph. Abbreviations: IFN, interferon; IL, interleukin; IP, inflammatory protein; LOD, limit of detection; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

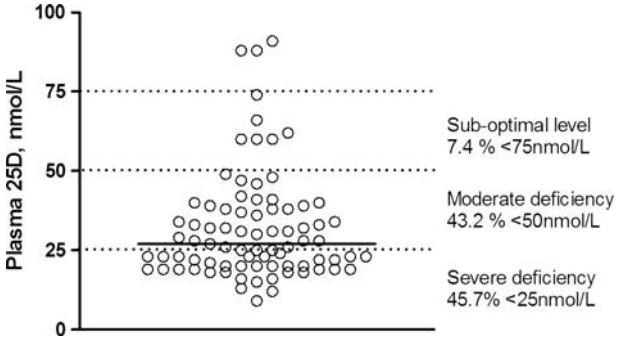

Figure 2. Baseline vitamin D status for all patients (n = 81). Dotted lines indicate the threshold for severe vitamin D deficiency (25(OH)D <25 nmol/L), moderate vitamin D deficiency (25(OH)D: 25–50 nmol/L), suboptimal 25(OH)D levels (25(OH)D: 50–75 nmol/L), and vitamin D sufficiency (25(OH)D >75 nmol/L).

and IL-18 during the first 2 weeks of ART was more pronounced in patients not on CTC (Supplementary Table 2).

We also compared the difference in cytokine concentrations between week 2 and baseline (delta change). Patients not on CTC who developed TB-IRIS had significantly increased IL-6, IL-8, IL-18, and TNF ($P \leq .012$). Conversely, in patients on CTC, there was only a trend for decreased IL-10 in patients who developed TB-IRIS ($P = .064$).

Relationship of Vitamin D Deficiency to TB-IRIS and Corticosteroid Therapy

Vitamin D deficiency was highly prevalent: only 3 of 81 patients (3.7%) had optimal vitamin D levels (Figure 2). Severe deficiency was observed in 37 of 81 patients (45.7%), moderate deficiency in 35 of 81 patients (43.2%) and the remaining 6 of 81 patients (7.4%) had suboptimal levels of 25(OH)D. CTC had no effect on baseline vitamin D status (Table 2).

We next analysed plasma 25(OH)D concentrations during the first 2 weeks of ART, stratified by CTC status (Figure 3). There was no baseline difference in 25(OH)D between patients who developed TB-IRIS and those who did not, irrespective of CTC use. However, 25(OH)D showed a slight, but statistically significant, decrease during the first 2 weeks of ART in those who developed TB-IRIS and did not receive CTC: from 24.7 nmol/L (IQR 19.7–37.2) at day 0 to 22.5 nmol/L (IQR 19.2–29.0) at 2 weeks, $P = .004$. At week 2 of ART, 25(OH)D was significantly lower in non-CTC TB-IRIS patients compared to non-IRIS patients: 22.5 nmol/L (IQR 19.2–29.0) vs 28.0 nmol/L (IQR 24.0–33.7), $P = .026$. Consistent with the limited change in cytokine profile in those who received CTC and developed TB-IRIS, these patients also had no significant change in 25 (OH)D levels at week 2 of ART.

Association Between 25(OH)D Deficiency and Plasma Cytokines

Due to the observation that non-CTC TB-IRIS patients showed a further decrease in 25(OH)D levels during ART and that these patients had a significant increase in the concentration of a number of cytokines/chemokines at the development of TB-IRIS, we next investigated if there was an association between severe vitamin D deficiency (<25 nmol/L) and plasma cytokine/chemokine concentrations in the non-CTC subgroup. Figure 4*A–D* shows the 4 cytokines that were associated with vitamin D status, in non-CTC patients. Patients who were severely vitamin D deficient had higher concentrations of IL-8 and IL-18 ($P \leq .038$) and a trend for higher IL-6 ($P = .052$) at baseline compared to non–severely deficient patients, and this difference was maintained at week 2 of ART for IL-8 ($P = .030$). There was a significant increase in plasma TNF and IL-8 in the severely vitamin D deficient patients during 2 weeks of ART ($P \leq .039$), and a trend for IL-6 ($P = .068$). At 2 weeks the number of TB-IRIS patients in

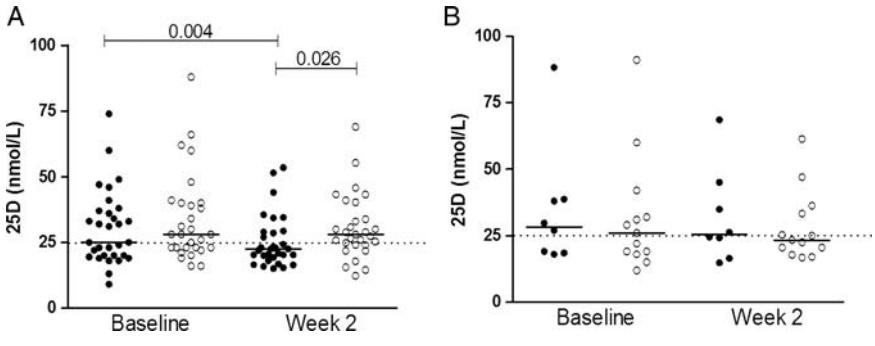

Figure 3. 25(OH)D concentration in patients who developed immune reconstitution inflammatory syndrome (IRIS; filled circles) or did not develop IRIS (open circles) at baseline and after 2 weeks on antiretroviral therapy (*A*) in patients who did not receive corticosteroids $n_{IRIS} = 31$, $n_{nonIRIS} = 29$ and (*B*) in patients prescribed corticosteroids $n_{IRIS} = 8$, $n_{nonIRIS} = 13$. Solid line at median; dotted line indicates severe vitamin D deficiency. Statistical test: Mann-Whitney (unpaired data) and Wilcoxon signed rank test (paired data); significant *P* values are indicated on the graph.

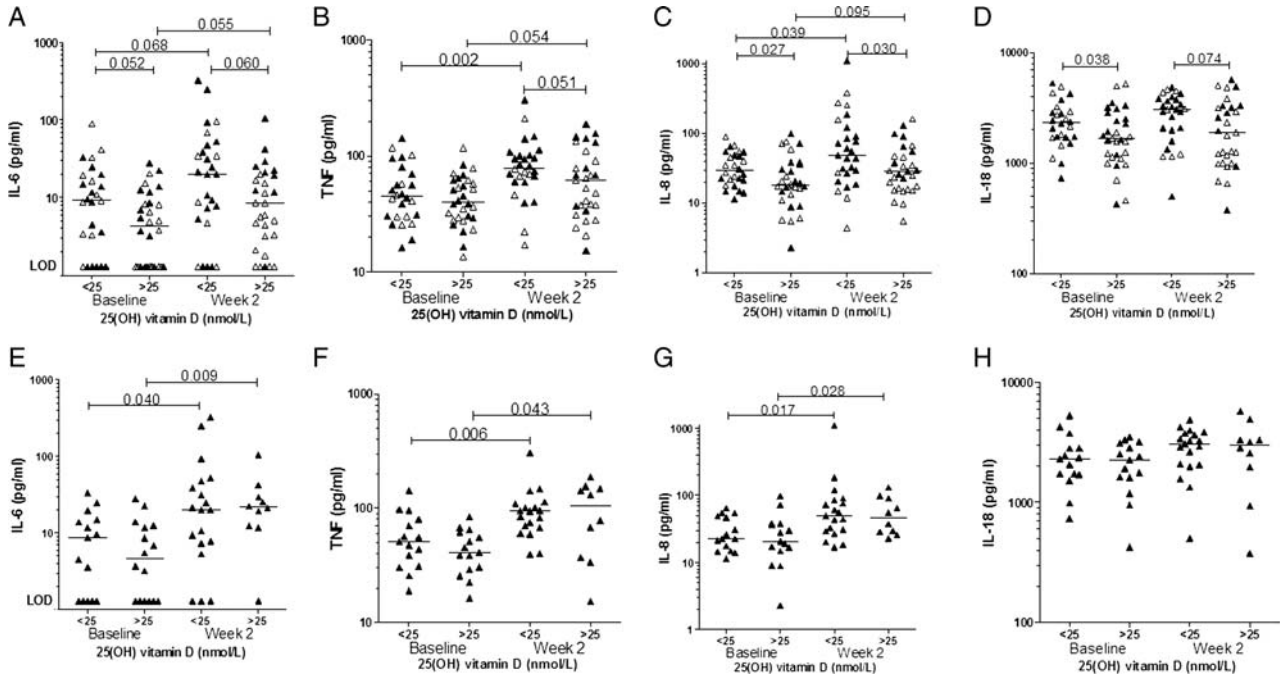

Figure 4. Cytokine concentrations stratified by severe vitamin D deficiency (25(OH)D <25 nmol/L, $n_{baseline} = 15$ immune reconstitution inflammatory syndrome (IRIS; filled triangles) and 11 non-IRIS (open triangles), $n_{week2} = 20$ IRIS and 7 non-IRIS) or nonsevere vitamin D deficiency (25(OH)D >25 nmol/L, $n_{baseline} = 15$ IRIS and 15 non-IRIS, $n_{week2} = 10$ IRIS and 19 non-IRIS) in patients who did not receive adjunctive corticosteroid therapy. Line at median. *P* values determined by Mann-Whitney test for medians. Abbreviations: IL, interleukin; LOD, limit of detection; TNF, tumor necrosis factor.

the severely vitamin D deficient group was proportionally higher than in the non–severely deficient group (20/27, 74% vs 10/29, 34.5%, $P = .003$). Therefore, we conducted the same analysis focusing on TB-IRIS patients only (Figure 4*E*–4*H*, not on CTC): there was no difference in cytokine levels between patients with 25(OH)D <25 nmol/L compared to >25 nmol/L, at both time points. However, IL-6, IL-8, and TNF significantly increased during ART in both groups. This suggests that vitamin D deficiency is likely to arise as a consequence of TB-IRIS, rather than the cause.

DISCUSSION

We confirmed in a longitudinal study that increased circulating cytokines/chemokines associate with TB-IRIS. We also report a very high prevalence of vitamin D deficiency in our prospective cohort of hospitalised HIV-1-TB coinfected patients in Cape Town. Although we found that vitamin D deficiency is not a risk factor for TB-IRIS development, the patients who develop TB-IRIS and do not receive CTC have a further reduction in circulating 25(OH)D levels in the first 2 weeks of ART, with lower 25(OH)D concentrations compared to non-IRIS, and a concomitant increase in circulating inflammatory cytokines/chemokines.

A recent study in HIV-1-infected women showed that 25 (OH)D sufficiency protected against all-cause mortality and HIV-1 disease progression [20]. Only 3.7% patients in our cohort had sufficient levels of 25(OH)D, comparable to the findings described in our recent study on vitamin D in Cape Town, not including IRIS patients [8]. Even taking into account a median hospitalisation time of 2 weeks before their baseline 25(OH)D was measured (which is less than the half-life of 25(OH)D [21]), suggests that at least 46% of patients severely deficient at this time, were also deficient upon hospitalization.

Because we previously demonstrated in a cross-sectional study that TB-IRIS associates with hypercytokinaemia [5], here we aimed to confirm this in a longitudinal study and determine when hypercytokinemia arose. As 26% of patients were on adjunctive CTC therapy for severe tuberculosis before starting ART, and CTC significantly reduced baseline plasma cytokine levels, we stratified our data according to CTC use. We found that 25(OH)D significantly decreased during the first 2 weeks of ART in patients not on CTC who developed TB-IRIS. Although the absolute magnitude of the drop in 25(OH)D was small, it may reflect a real biological phenomenon. We and others have previously demonstrated a decrease in 25(OH)D concentrations in HIV-infected persons

on NNRTI-based regimens [22–24]. In the present study, all patients were on NNRTI-based regimen as well as on tuberculosis treatment. The fall in plasma 25(OH)D levels observed in this study could be partly attributable to the interference of ART and anti-tuberculosis therapy with vitamin D metabolism [25, 26].

Various explanations have been proposed as to why HIV-infected persons have lower vitamin D status [27]: (1) increased use of 25(OH)D for maturation and proliferation of T lymphocytes during HIV infection; and (2) increased cytokine levels, specifically TNF, blocking the stimulatory effect of parathyroid hormone on the production of the hormonally active vitamin D (1,25-dihydroxyvitamin D, $1,25(OH)_2D$). Although these explanations may account for the drop in 25(OH)D, it is also possible that ART initiation in a patient who still has high tuberculosis antigen load, despite starting antituberculosis therapy over activates macrophages and dendritic cells to produce $1,25(OH)_2D$ locally, stimulated by the reconstituting T helper cell-produced IFN-γ [11]. Interestingly, baseline IFN-γ was significantly higher in TB-IRIS compared to non-IRIS patients not on CTC. Increased production of $1,25(OH)_2D$ will then induce catabolism of 25(OH)D and $1,25(OH)_2D$ through their catabolising enzyme CYP24A1. This phenomenon has been used to explain the low 25(OH)D associated with granulomatous diseases where activated macrophages convert 25(OH)D to $1,25(OH)_2D$ [19]. Confirmation of this hypothesis would be measuring $1,25(OH)_2D$ and vitamin catabolites in plasma, resulting from systemic spillover, but this was not performed in our study due to limited sample volume.

We found that TB-IRIS was associated with lower 25(OH)D and higher cytokine concentrations 2 weeks following ART in those not receiving CTC and that these differences did not exist at baseline. The increased concentrations of IL-6, IL-8, IL-18, and TNF at TB-IRIS presentation are in line with our previous cross-sectional study [5] and also support the role for neutrophils (IL-8) and natural killer (NK) cells (IL-18) in the pathogenesis of IRIS [28]. Moreover, we showed that IL-8 and IL-18 concentrations were significantly higher in severely 25(OH)D deficient patients at baseline. However, although Figure 4 shows a correlation between high cytokine levels and lower vitamin D in the whole cohort, there is no correlation between severe vitamin D deficiency and hypercytokinemia within TB-IRIS patients. This suggests that the rise in cytokines in TB-IRIS patients is not due to decreased 25(OH)D after starting ART. In fact, it may be that lower 25(OH)D in IRIS patients is a consequence of the combination of 25(OH)D consumption during hypercytokinemia and an interaction between tuberculosis therapy and ART with vitamin D metabolism. Thus, although decreased 25(OH)D may not be the cause of TB-IRIS, it may contribute to the continued hyperinflammatory response. Indeed, $1,25(OH)_2D$ has been shown to inhibit neutrophil chemotaxis, degranulation, and oxidative burst in response to chemokines such as IL-8, via the complement fragment C5a [29, 30]. Moreover, $1,25(OH)_2D$ inhibits the production of proinflammatory cytokines via inhibition of intracellular pathways, such as the p38 signaling for IL-6 or the NF-κB complex pathway for IL-8 [31]. Therefore, a continued increase in neutrophil activation and cytokine production would be seen in absence of sufficient conversion of 25(OH)D toward $1,25(OH)_2D$.

Finally, the different immunological profiles observed between those who received corticosteroids compared to those who did not, suggests there might be different immunological triggers leading to TB-IRIS development. From all patients receiving CTC pre-ART, 38% developed TB-IRIS despite decreased CTC-induced plasma cytokine concentrations. Clinically, there was a trend toward less severe clinical manifestation of TB-IRIS in these patients but longer time of hospitalization from the start of ART. The latter could be explained by the fact that CTC was mainly prescribed to patients requiring intensive medical care. Future studies should therefore stratify patients according to CTC use, when investigating immunological correlates of IRIS risk and disease manifestation.

In conclusion, we found vitamin D deficiency not to be a risk factor for developing TB-IRIS, but patients developing TB-IRIS become more severely vitamin D deficient after 2 weeks of ART, while they also develop hypercytokinemia. Due to an association between IL-6, IL-8, and IL-18 with vitamin D status at baseline, together with the in vitro evidence of antiinflammatory and the antimycobacterial properties of vitamin D, the role of 25(OH)D and its active form $1,25(OH)_2D$ should be further elucidated in TB-IRIS. Future work should identify what is triggering the reduction in 25(OH)D, whether this correlates with increased $1,25(OH)_2D$ and whether 25(OH)D reduction occurs before or after hypercytokinemia and the onset of IRIS symptoms. An approach would be to supplement patients with vitamin D prior to initiation of ART and to determine if this decreases TB-IRIS incidence.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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