High-dose vitamin D_3 reduces deficiency caused by low UVB exposure and limits HIV-1 replication in urban Southern Africans

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Cape Town, South Africa, has a seasonal pattern of UVB radiation and a predominantly dark-skinned urban population who suffer high HIV-1 prevalence. This coexistent environmental and phenotypic scenario puts residents at risk for vitamin D deficiency, which may potentiate HIV-1 disease progression. We conducted a longitudinal study in two ethnically distinct groups of healthy young adults in Cape Town, supplemented with vitamin D_3 in winter, to determine whether vitamin D status modifies the response to HIV-1 infection and to identify the major determinants of vitamin D status (UVB exposure, diet, pigmentation, and genetics). Vitamin D deficiency was observed in the majority of subjects in winter and in a proportion of individuals in summer, was highly correlated with UVB exposure, and was associated with greater HIV-1 replication in peripheral blood cells. High-dosage oral vitamin D_3 supplementation attenuated HIV-1 replication, increased circulating leukocytes, and reversed winter-associated anemia. Vitamin D_3 therefore presents as a low-cost supplementation to improve HIV-associated immunity.

seasonal variation | infectious disease | polymorphism | pigmentation | nutrition

Vitamin D is recognized as having diverse physiological and immunomodulatory functions, and deficiency is associated with a range of communicable and noncommunicable diseases, including HIV/AIDS progression and mortality (1, 2). Previtamin D_3 is made in skin when UVB photons react with 7-dehydrocholesterol (7-DHC) in the cell membranes of keratinocytes (3). Constitutive and facultative skin pigmentation regulates the penetration of UV radiation into the skin, because eumelanin competes with 7-DHC for UVB photons, thus controlling the availability of UVB for previtamin D_3 production (4). Therefore people with high eumelanin content (and thus darker skin) require greater UVB exposure to make previtamin D_3 and are more prone to vitamin D deficiency. This requirement for greater exposure is exacerbated by seasonal UVB fluctuation; generally UVB levels exhibit a winter decline at latitudes >30° (5, 6).

Serum vitamin D [25-hydroxy-vitamin D, 25(OH)D] levels are determined by skin (dermal and epidermal) production, dietary intake, storage, and turnover. Each of these determinants is modified at a variety of levels: skin production by pigmentation, age, and the time, duration, and level of UVB exposure; dietary intake by vitamin D composition of foods; storage by body mass index (BMI) (7) and serum vitamin D-binding protein (DBP) (8); and turnover by polymorphic variation in genes encoding metabolizing enzymes [cytochrome P450 (CYP) 2R1, CYP27B1, CYP24A1, 7-DHC reductase (DHCR7)], the vitamin D receptor (VDR) and DBP (9–12), age (13), infection (14, 15), serum calcium and parathyroid hormone concentrations (16), and smoking habit (17). The two most significant determinants are UVB exposure and dietary intake, and although deconvolution of their relative impact is vital for understanding how to maintain vitamin D sufficiency for disease prevention, they are rarely investigated in the same study, nor are results adjusted for all other confounders. Furthermore, the majority of studies investigate vitamin D status in relation to chronic disease; there is a dearth of information regarding the determinants of vitamin D sufficiency in the healthy state and its relation to disease prevention.

Cape Town has a diverse population with respect to ethnicity, skin pigmentation, and socioeconomic status. The Xhosa, who migrated as part of the geographic expansion of north and northwest African agriculturalists, are a significant proportion of the population. Another major population group comprises people of self-identified Cape Mixed ancestry, who represent a complex admixture of Xhosa, Khoisan (the oldest inhabitants), European, South Asian, and Indonesian populations (18). Given their exposure to seasonal UVB variation and high infectious disease risk, the peoples of the Cape are of particular importance in studying the determinants and immunological consequence of vitamin D status. In the context of greatest HIV-1 risk, those of particular importance are females aged 15–24 y and males aged 20–29 y (19).

We thus undertook a longitudinal study of healthy young adults in Cape Town to assess the relative contribution of pigmentation, seasonal UVB exposure, dietary vitamin D intake,

Significance

Vitamin D deficiency is associated with HIV/AIDS progression and mortality. Seasonal decline in UVB radiation, darkly pigmented skin, low nutritional vitamin D intake, and genetic variation can increase risk of deficiency. Cape Town, South Africa, has a seasonal UVB regime and one of the world's highest rates of HIV-1 infection, peaking in young adults. In two ethnically distinct groups of young adults in Cape Town we found high prevalence of seasonal vitamin D deficiency resulting from inadequate UVB exposure. This deficiency was associated with increased permissiveness of blood cells to HIV-1 infection which was reversed by vitamin D_3 supplementation. Vitamin D may be a simple, cost-effective intervention, particularly in resource-poor settings, to reduce HIV-1 risk and disease progression.

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genetic variation, serum DBP, and smoking habit on vitamin D status and investigated whether seasonal vitamin D variation and vitamin D supplementation impact HIV-1 immunity in this high-risk population.

Results

Highly Prevalent Seasonal Vitamin D Deficiency and Its Reversal by Winter Supplementation. One hundred healthy (asymptomatic, BMI <30) young (18- to 24-y-old) adults (Xhosa, $n = 50$; Cape Mixed, $n = 50$) were recruited from two neighboring districts of Cape Town in the summer and were reassessed in the winter (for loss to follow-up, see Fig. S1). In winter, all participants received cholecalciferol (50,000 IU) weekly for 6 wk, and 30 Xhosa participants were followed up for 6 wk after their winter visit (Fig. 1*A*). All enrolled participants were asymptomatic with no evidence of infection (Fig. S2 *A–C*). The populations were well matched with similar female:male ratios and only a small difference in age (Xhosa 21 y vs. Cape Mixed 18.5 y; $P < 0.0001$) and smoking status (Xhosa 50% vs. 18%; $P = 0.0014$) (Table S1). Xhosa participants had darker skin pigmentation as measured by upper inner arm and forehead melanin index (MI) and erythema index (EI, a measure of tanning) ($P < 0.0001$; Table S2).

Although their higher melanin content reduced the rate of skin vitamin D production, Xhosa participants actually had higher serum 25(OH)D levels in summer than Cape Mixed participants (median 72.6 vs. 65.5 nmol/L; $P = 0.038$, Table 1). Cape Mixed participants also had a trend toward greater vitamin D deficiency (<50 nmol/L) in summer (16 vs. 4%; $P = 0.077$). Conversely, there was no difference in 25(OH)D levels between population groups in winter, when a significant drop in 25(OH)D levels was observed in both populations ($P < 0.0001$) and the majority of participants became vitamin D deficient (Fig. 1*B*). Severe deficiency (<30 nmol/L) occurred in 18% of Xhosa participants and 12% of Cape Mixed participants in the winter, and overall 64% and 70%, respectively, had deficient serum 25(OH)D levels (Table 1). After winter supplementation, 77% of the Xhosa group gained optimal levels (≥75 nmol/L) [median126.4 nmol/L, interquartile range (IQR) 74.631–57.1 nmol/L] (Fig. 1*C*), and there was no change in corrected serum calcium (winter mean ± SD 2.31 ± 0.08 mmol/L vs. postsupplementation 2.34 ± 0.09 mmol/L) (Fig. S2*D*). Cape Mixed females had lower 25(OH)D levels in winter than Cape Mixed males (median 41.46 vs. 50.80 nmol/L; $P = 0.0054$), and Xhosa females had lower 25(OH)D levels after supplementation than Xhosa males (median 113.3 vs. 147.9 nmol/L, $P = 0.047$), indicating that females in both groups are at risk for lower 25(OH)D (Fig. 1 *D* and *E*).

Personal UVB Exposure, but Not Diet, Varies by Season. Dietary intake of vitamin D was estimated using a 7-d quantitative food frequency questionnaire administered at each study visit. According to the estimated average requirement (EAR) cutoff-point method, 78–88% of participants in both populations had intakes below the EAR (400 IU) throughout the year, with median intakes ranging from 170 to 235 IU across groups and seasons (Fig. 1*F* and Table 1). There was no difference in intake between sexes in the Xhosa participants, but Cape Mixed females had lower intakes than Cape Mixed males in both seasons ($P < 0.001$), mirroring the sex patterns observed for 25(OH)D levels (Fig. 1 *E*, *G*, and *H*).

To understand the extent to which UVB exposure contributes to vitamin D deficiency, solar UVB was monitored daily for the duration of the study, and participants completed sun-exposure questionnaires. Both groups spent significantly longer in the sun in summer than in winter ($P \leq 0.014$), with personal net UVB (PNUVB) exposure more than 10-fold higher in summer ($P < 0.0001$, Fig. 1*I*). Xhosa participants spent ∼4 h longer each week in the sun in both seasons than Cape Mixed participants (median: 1,335 vs. 1,096 minutes in summer and 795 vs. 540 minutes in winter) (Table S2), and they exposed larger areas of the body in the summer (median 30.0 vs. 22.6%; $P = 0.0002$). Both groups reduced their body exposure in winter to similar levels (12.5%), although the relative winter decrease was greater for Xhosa participants. There was limited sunscreen use in both groups (Table S2). Thus, Xhosa participants may partly compensate for their darker skin pigmentation by increasing their PNUVB in the summer (median 42,982 vs. 20,603 J; $P < 0.0001$), which is reflected in higher 25(OH)D levels in summer in Xhosa participants than in Cape Mixed participants (Table 1). However, Xhosa participants did not maintain higher UVB exposure in the winter (PNUVB 1,304 J vs. 1,450 J; $P = 0.63$), and they were at greater risk of deficiency, with 18% of participants exhibiting severe deficiency.

Genetic Variation Has a Larger Effect on the Response to Supplementation than on Seasonal Deficiency. To investigate the effects of genetic variation on serum 25(OH)D concentrations, 10 SNPs in six genes associated with vitamin D deficiency were genotyped in all participants: *DBP* (rs7041 and rs4588), *DHCR7* (7-DCH reductase

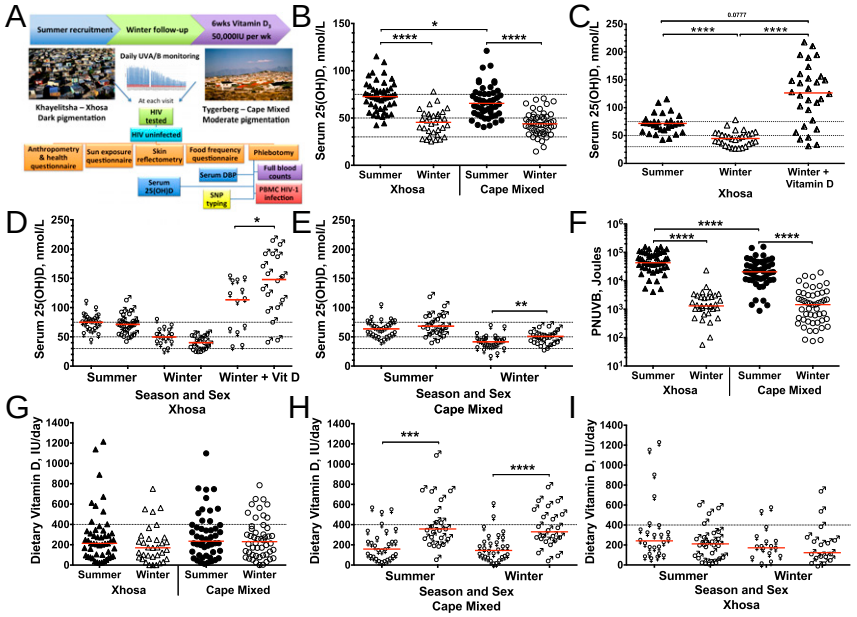

Fig. 1. Vitamin D status, dietary vitamin D intake, and personal UVB exposure of Xhosa and Cape Mixed participants in Cape Town, South Africa, in summer, winter, and after receiving vitamin D_3 in winter. (*A*) Study design. (*B* and *C*) Serum 25(OH)D concentration stratified by season (*B*) and after receiving vitamin D_3 (*C*). Dotted lines indicate status thresholds: insufficiency, <75 nmol/L; deficiency, <50 nmol/L; severe deficiency, <30 nmol/L. (*D* and *E*) Serum 25(OH)D concentration stratified by sex (female, ♀; male, ♂) in Xhosa (*D*) and Cape Mixed (*E*) participants. (*F*) Dietary vitamin D intake measured by the food frequency questionnaire. Dotted lines indicate EAR. (*G* and *H*) Dietary vitamin D intake stratified by sex in Xhosa (*G*) and Cape Mixed (*H*) participants. (*I*) PNUVB. Xhosa: summer, $n = 50$; winter $n = 33$; winter + vitamin D, $n = 30$; Cape Mixed: summer and winter, $n = 50$. Medians are indicated by red lines. Significance was tested by the Wilcoxon rank test between seasons, by the Friedman test with Dunn's multiple comparisons test for 25(OH)D postsupplementation ($n = 30$), and by the Mann–Whitney test between populations and sex; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Table 1. Serum 25(OH)D, dietary vitamin D intake, and UVB exposure by season and population

Measure	Summer	Winter	*P* value*
Serum 25(OH)D, median nmol/L (range): Xhosa	**72.6 (62.1–80.43)**†	45.4 (35.7–51.2)	**<0.0001**
Serum 25(OH)D, median nmol/L (range): Cape Mixed	**65.5 (54.6–76.1)**	43.8 (33.5–54.2)	**<0.0001**
Vitamin D status, nmol/L (%)			**<0.0001**
Xhosa: Severe deficiency, <30 nmol/L	0	6 (18)	
Xhosa: Deficiency, <50 nmol/L	2 (4)	21 (64)	
Xhosa: Insufficiency, 50–75 nmol/L	28 (56)	11 (33)	
Xhosa: Sufficiency, >75 nmol/L	20 (40)	1 (3)	
Cape Mixed: Severe deficiency, <30 nmol/L	0	6 (12)	
Cape Mixed: Deficiency, <50 nmol/L	8 (16)	35 (70)	
Cape Mixed: Insufficiency, 50–75 nmol/L	27 (54)	15 (30)	
Cape Mixed: Sufficiency, >75 nmol/L	15 (30)	0	
Dietary vitamin D intake, median IU/d (IQR): Xhosa	213 (94–335)	170 (68–266)	0.26
Dietary vitamin D intake, median IU/d (IQR): Cape Mixed	235 (120–395)	230 (99–369)	0.24
Personal weekly UVB, median J (IQR): Xhosa	**42,982 (28,651–90,552)**†	1,304 (875.5–2,878)	**<0.0001**
Personal weekly UVB, median J (IQR): Cape Mixed	**20,603 (10,887–42,897)**	1,450 (392.6–3,530)	**<0.0001**

*Wilcoxon rank test or Fisher's exact test between seasons, bold type indicates statistically significant difference between summer and winter values.
†Mann–Whitney test significant between populations, $P < 0.04$, bold type indicates statistically significant difference between summer and winter values.

and rs12785878), *CYP2R1* (vitamin D 25-hydroxylase and rs10741657), *CYP24A1* (vitamin D 24-hydroxylase and rs6013897), *CYP27B1* (vitamin D 1α-hydroxylase and rs10877012), and *VDR* (rs2544037, rs10783219, rs10735810, and rs731236) (9–11, 20). All were in Hardy–Weinberg equilibrium, and five SNPs (*CYP27B1* rs10877012; *VDR* rs10783219 and rs731236 *TaqI*; and *DBP* rs7041 and rs4588) had significantly higher minor allele frequency (MAF) in the Cape Mixed participants ($P < 0.03$, Table 2). *DBP* rs7041 and rs4588 are combined to form the Group component (Gc) haplotype of which there are three major alleles, Gc1F, Gc1S, and Gc2, with the Gc1F protein having higher binding affinity for serum 25(OH)D (8). Xhosa participants had significantly higher frequency of Gc1F/Gc1F carriers (76 vs. 34%), whereas the most common haplotype combination in the Cape Mixed participants was Gc1F/Gc1S (38 vs. 18%; $P = 0.0002$) (Table 2). Serum DBP levels also were measured in all participants; there was no effect of season, vitamin D supplementation or Gc haplotype on serum DBP levels in either population, but Cape Mixed participants had lower median levels in both seasons (summer/winter:106/107 vs. 125/127 μg/mL; $P \leq 0.0092$) (Fig. S2 *E* and *F*).

Although the powering was modest, genotypes were added to the stepwise regression and general linear models (GLM) for determinants of total 25(OH)D (Table 3 and Tables S3 and S4). In exploratory analyses of combined participants, a greater genotypic effect was observed after supplementation than with seasonal variation; those heterozygous for *VDR FokI* (rs10735810) had lower 25(OH)D in the winter and postsupplementation ($P < 0.04$); those heterozygous for *CYP24A1* rs6013897 had lower

Table 2. SNP frequency in Xhosa and Cape Mixed participants

Gene and SNP (also known as)	Location (function)	Allele*	Xh (Major)	Xh (Het)	Xh (Minor)	CM (Major)	CM (Het)	CM (Minor)	*P*-value†
CYP2R1 rs10741657 (rs2060793)‡	Promoter	A/G	0.72	0.22	0.06	0.54	0.38	0.08	0.17
CYP27B1 rs10877012 (rs4646536)‡	5′ UTR	C/A	0.86	0.12	0.02	0.54	0.42	0.04	**0.002**
CYP24A1 rs6013897	3′ downstream	A/T	0.52	0.42	0.06	0.66	0.26	0.08	0.24
DBP									
rs7041 (Asp416Glu)	Exon 11 (nonsyn)	C/A§	0.8	0.18	0.02	0.5	0.44	0.06	**0.007**
rs4588 (Thr420Lys)	Exon 11 (nonsyn)	T/G§	0.96	0.04	0	0.78	0.2	0.02	**0.027**
VDR									
rs2544037	Promoter	A/G	0.52	0.38	0.1	0.58	0.3	0.12	0.7
rs10783219	Intron 0	A/T	0.9	0.1	0	0.72	0.22	0.06	**0.044**
rs10735810 (*FokI*)	Exon 3 (nonsyn)	A/G	0.64	0.3	0.06	0.56	0.38	0.06	0.69
rs731236 (*TaqI*)	Intron 9	A/G	0.72	0.28	0	0.54	0.4	0.06	0.069
DHCR7 rs12785878 (rs7944926; rs3794060)‡	Intron 2	G/T§	0.52	0.42	0.06	0.5	0.44	0.06	0.98
DBP Gc Haplotype (alleles rs7041–rs4588)¶									
Gc1F/Gc1F	Exon 11 (nonsyn)	CC-TT	0.76			0.34			
Gc1F/Gc2	Exon 11 (nonsyn)	CC-TG	0.04			0.14			
Gc2/Gc2	Exon 11 (nonsyn)	CC-GG	0			0.02			
Gc1F/Gc1S	Exon 11 (nonsyn)	CA-TT	0.18			0.38			
Gc2/Gc1S	Exon 11 (nonsyn)	CA-GT	0			0.06			
Gc1S/Gc1S	Exon 11 (nonsyn)	AA-TT	0.02			0.06			

CM, Cape Mixed; Het, heterozygous; Xh, Xhosa.
*Major/minor alleles and frequencies.
†*P* values for differences between sites tested by χ^2 test for trend, bold type indicates statistically significant difference in SNP frequencies.
‡In linkage disequilibrium, $r^2 = 1.00$ (9, 10).
§On reverse strand.
¶Predicted according to ref. 21.

25(OH)D levels postsupplementation ($P = 0.006$); and those heterozygous for *CYP2R1* rs10741657 and *DBP* rs7041 had higher 25(OH)D levels postsupplementation ($P = 0.002$) (Fig. S3).

Personal UVB Exposure Is the Major Determinant of Vitamin D Status. We next used regression models applied to all variables (*SI Materials and Methods*) to identify the determinants of serum 25(OH)D in both groups. Stepwise regression (Table S3) identified PNUVB as the dominant determinant ($F = 123.2$, $P < 0.0001$), followed by area of skin exposed ($F = 10.7$, $P = 0.0013$) and arm MI ($F = 7.40$, $P = 0.0072$). *DBP* haplotype Gc1F/Gc1S and two *VDR* SNPs, *TaqI* rs731236 and rs2544037, also contributed to the model, as did duration of UVB exposure and skin redness (arm EI), to a lesser extent. To determine the directionality of effect a GLM approach was applied adjusting for age and smoking status (Table 3). Again PNUVB was the dominant determinant, followed by area of skin exposed and weekly duration of exposure, all positively contributing to serum 25(OH)D, whereas *VDR FokI* rs10735810 AG and Cape Mixed ancestry where negatively correlated with 25(OH)D. The two measures of skin redness (arm and forehead EI), as well as Gc1F/Gc1S, *VDR FokI* rs10735810 AA, and *DBP* rs7041 CA, positively contributed to serum 25(OH)D.

The determinants of severe deficiency in winter and sufficiency in the summer also were examined using a GLM approach (Table S4). Sunlight exposure as a component of PNUVB did not prevent serious deficiency in the winter, but the area exposed and duration were important. The UVB content of winter sunlight is weak, but individuals who were in the sun for longer periods and who had the most surface area exposed produced more vitamin D. Darker skin (higher arm and forehead MI) also was associated with lower serum 25(OH)D status. Sex had an effect but correlated with area exposed and duration of exposure. Sufficiency in the summer was affected most strongly by duration of exposure and PNUVB and to a lesser extent by the degree of skin redness (arm EI). Possession of the *DHCR7* rs12785878 GG genotype had a small effect on optimal serum 25(OH)D, but no other genes had a significant effect. Smoking status and sex also were influential in summer but only through correlation with patterns of sun exposure and skin pigmentation, respectively.

Winter Vitamin D_3 Supplementation Increases Peripheral WBC Count and Counteracts Anemia. To investigate the functional consequence of seasonal serum 25(OH)D levels on the immune system, we next investigated seasonal differences in full blood count (FBC) and the effect of vitamin D_3 supplementation on FBC in Xhosa participants. Vitamin D_3 supplementation in the winter increased WBC count ($P = 0.0016$) and in particular lymphocyte count ($P = 0.023$), and there was a winter trend for decreased monocytes (Fig. 2 *A–C*). In the winter, participant's RBC parameters tended toward macrocytic anemia [evidenced by decreased RBC and RBC distribution width (RDW), increased mean corpuscular volume, and a trend toward decreased Hb], and this tendency was reversed by supplementation ($P \leq 0.0007$) (Fig. 2 *D–F* and Fig. S4*A*). The winter decline in RBC, RDW, and Hb also was seen in participants with Cape Mixed ancestry, although the effect of supplementation was not measured (Fig. S4 *B–D*).

Table 3. Determinants of serum 25(OH)D concentration

Variable	*t*-statistic	*R*-statistic	*P* value*
PNUVB	9.502	0.579	<0.0001
Area of skin exposed	5.855	0.401	<0.0001
Weekly duration of UVB	4.401	0.312	<0.0001
Arm EI	3.617	0.261	0.0004
Gc1F/Gc1S	3.026	0.221	0.0028
VDR rs10735810 AA	2.715	0.199	0.0073
DBP rs7041 CA	2.656	0.195	0.0086
Forehead EI	2.368	0.174	0.0190
Cape Mixed ancestry	−2.271	−0.167	0.0243
VDR rs10735810 AG	−2.544	−0.187	0.0118

*P values derived using linear regression, with adjustment for covariates (smoking and age). The false-discovery rate was determined by the Benjamini–Hochberg; all <0.1.

Winter Vitamin D_3 Supplementation Decreases HIV-1 Replication in Peripheral Blood Mononuclear Cells. Because supplementation modified peripheral WBC and RBC counts, we next investigated the functional consequences on response to HIV-1 infection. The active vitamin D metabolite, 1α,25-dihydroxy-vitamin D, has been shown in vitro to inhibit HIV-1 replication in macrophages via induction of autophagy, mediated via cathelicidin induction (22), and 25(OH)D deficiency is associated with HIV-1 progression (1). Therefore we investigated the functional consequences of seasonal variation in serum 25(OH)D levels and winter vitamin D_3 supplementation on the extent of HIV-1 replication in freshly isolated peripheral blood mononuclear cells (PBMCs), at each study visit. PBMCs were cultured in the presence of fresh 20% autologous serum, isolated at the same time as PBMCs, to maintain the in vivo cellular environment as best possible, with regard to seasonal serum 25(OH)D levels, autologous DBP, and other circulating chemokines/cytokines. Two preparations of HIV-1 BaL, purified and unpurified, were used for infection. Unpurified HIV-1 preparations contain exosomes, microvesicles, and conditioned medium from propagation and were tested for the likelihood that activating cells might result in greater productive infection of unstimulated cells.

Infection of PBMCs in winter, compared with summer, resulted in greater productive HIV-1 infection on day 9, as measured by culture supernatant p24 antigen levels. This result was seen in PBMCs isolated from both Xhosa ($P = 0.0003$, Fig. 2 *G–I*) and Cape Mixed ($P < 0.0001$, Fig. S4 *E* and *F*) participants. This winter increase in HIV-1 infection was seen with both preparations of HIV-1, with ∼1-log higher p24 measured from cells infected with unpurified virus (Xhosa median 7,038 pg/mL unpurified vs. 738 pg/mL purified) (Fig. 2 *H* and *I*).

After 6 wk of vitamin D_3 supplementation in winter, the winter increase in HIV-1 p24 was attenuated, and Xhosa participants' PBMCs showed a diminished capacity for productive HIV-1 infection: on day 9 p24 levels had dropped to the level as observed in summer (Fig. 2*G*). Again, this decrease occurred with infections using both purified and unpurified virus (Fig. 2 *H* and *I*), demonstrating the robustness of oral vitamin D_3 supplementation in suppressing productive HIV-1 infection in peripheral blood cells ex vivo. Moreover there was a significant negative correlation between paired serum 25(OH)D and day 9 p24 concentrations from PBMCs infected with purified virus, across all time points (Spearman $r_s = -0.36$; $P < 0.0001$), indicating a direct correlation between serum 25(OH)D levels and the ability of peripheral blood cells to limit productive HIV-1 infection.

Discussion

The multitude of, and complex interactions among, variables that modify vitamin D status and their impact on immunological function are poorly understood, particularly in the context of disease prevention in healthy individuals. In an urban African location with seasonal UVB variability and high infectious disease prevalence, we found that personal UVB exposure habit is the most important determinant of vitamin D status in healthy adults with moderate to dark skin pigmentation. Moreover, we found that the reversal of vitamin D deficiency in winter through oral supplementation can modulate the number and function of circulating WBCs, prevent anemia, and decrease productive HIV-1 infection. Furthermore, we found that UVB exposure habit can counterbalance the effects of pigmentation, genetic variation, and poor dietary intake.

The skin of the indigenous people of the Cape, the Khoisan, is considerably lighter than that of either study group (23) and may represent a long-established adaptation to seasonal UVB. The darker skin of both study populations denotes a degree of

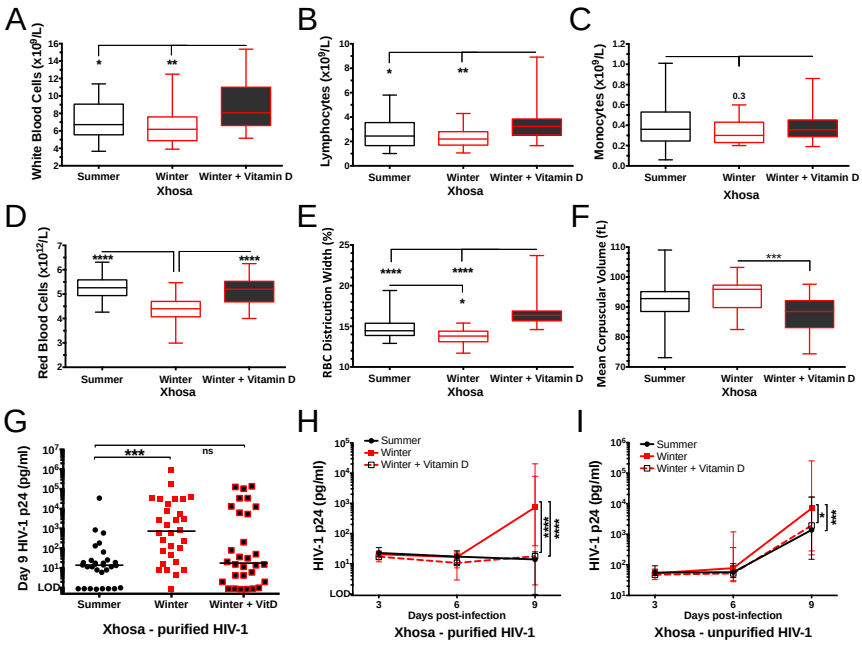

Fig. 2. FBC and HIV-1 replication in PBMCs from Xhosa participants in summer, winter, and after receiving winter vitamin D supplementation. (*A–F*) Box plots show WBC (*A*), lymphocyte (*B*), monocyte (*C*), and RBC (*D*) counts, RBC distribution width (*E*), and mean corpuscular volume (*F*) measured at each study visit (summer, $n = 42$; winter, $n = 23$; winter + vitamin D, $n = 30$). The lines across the box plots indicate the median (minimum–maximum); Kruskal–Wallis with Dunn's multiple comparison test. (*G*) HIV-1 p24 concentration in culture supernatant 9 d postinfection of PBMCs ($n = 30$ longitudinally; the line indicates the median) with purified HIV-1 on the day of phlebotomy in 20% autologous serum; Friedman test with Dunn's multiple comparison test. (*H* and *I*) HIV-1 p24 concentration in culture supernatant on day 3, 6, and 9 postinfection of PBMCs [$n = 30$ longitudinally, median (IQR)] with purified HIV-1 (*H*) or unpurified HIV-1 (*I*); repeated measures two-way ANOVA with Tukey's multiple comparison test following $\log_{10}$ transformation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. LOD, limit of detection (1 pg/mL); ns, not significant.

mismatch between skin pigmentation and environmental UVB resulting from their recent migration into the region; this effect is exacerbated by wearing concealing clothing and indoor living in the winter. The high prevalence of vitamin D deficiency in the winter in both groups indicates that people with moderate or dark pigmentation are at high risk of deficiency in the absence of significant dietary vitamin D intake when UVB radiation is limited by seasonal fluctuations.

We also noted significant polymorphic variation between the two populations for 5 of the 10 vitamin D-associated SNPs investigated, contributing further genetic insight into these understudied populations. Given the low MAF of the SNPs investigated, particularly in the Xhosa population, we identified only a minor effect of genetic variation on seasonal vitamin D status. This finding mirrors the recent genomewide association study in 16,125 individuals, from five cohorts, which showed the proportion of variation in 25(OH)D attributable to genetic variation was 1–4% (9). However, despite the small sample size, we identified four SNPs, in *VDR*, *CYP24A1*, *CYP2R1*, and *DBP*, which modify the response to high-dose supplementation. Although these results need to be confirmed in larger cohorts, such associations are supported by our previous finding that polymorphic variation in *VDR* modifies the effect of high-dose vitamin D supplementation on hastening time to sputum culture conversion in TB patients receiving intensive-phase treatment (24).

In South Africa, young females are at the greatest risk of HIV-1 infection (19). We found females in both populations also were at greater risk of deficiency, indicating this is an important target group for intervention. Dietary intake played a greater role in maintaining serum 25(OH)D levels when UVB levels were low. However, it is unlikely that a diet richer in vitamin D-containing foods alone can compensate for the seasonal changes in insolation and UVB at this or higher latitudes, especially when colder outdoor temperatures and the associated wearing of clothes and indoor living reduce the likelihood of exposure to weak UVB-containing sunlight. Recommendations for personal vitamin D supplementation or wide-scale food fortification may be considered, along with season-specific recommendations for short-duration sun exposure around noon in the winter and between ∼1.0 and 1.5 h sun exposure in the spring and autumn. Short periods of UVB exposure around solar noon are highly effective in raising serum 25(OH)D levels and pose a low risk to general and skin health (25).

Our findings of decreased winter WBC counts, particularly lymphocytes, and an increase in numbers following vitamin D supplementation corroborate a small longitudinal study in healthy Scandinavian adults with light pigmentation, which found a similar decrease in lymphocytes in winter, particularly $CD4^+$ and $CD8^+$ T cells, which was associated with reduced 25(OH)D (26). Higher serum 25(OH)D levels in children initiating antiretroviral therapy (ART) also were associated with higher $CD4^+$ T-cell restoration (27). Further studies will investigate the detailed changes in innate and adaptive immune cell populations in our cohorts. Vitamin D deficiency also has been associated previously with anemia and low Hb in HIV-infected women (1). We found that vitamin D supplementation reversed winter-associated macrocytic anemia, suggesting that this adjunct therapy also may be effective in preventing anemia in HIV-infected individuals.

The demonstration that high-dosage oral vitamin D supplementation reversed serum 25(OH)D deficiency and attenuated the seasonal increase in ex vivo HIV-1 replication, similar to our previous finding that oral vitamin D reduces *Mycobacterium tuberculosis* replication in whole blood (28), provides strong evidence for the positive preventative effects of vitamin D supplementation for people with vitamin D deficiency and serious infectious diseases, conditions which apply to many cities in which the prevalence of vitamin D deficiency continues to rise. Furthermore, vitamin D may be a simple, cost-effective intervention, particularly in resource-poor settings, to prevent disease progression in persons infected with HIV-1 by suppressing viral replication, raising peripheral lymphocyte counts, and preventing anemia, potentially prolonging the time to ART initiation and enhancing the beneficial effects of ART once initiated.

Materials and Methods

Study Design. The study was conducted in accordance with the Helsinki 1964 declaration, including subsequent revisions, and the South African Guidelines for Good Clinical Practice and the Medical Research Council Ethical Guidelines for Research. Ethical approval was received from the Human Research Ethics Review Boards of the Faculty of Health Sciences, University of Cape Town (ref. 003/2013) and the Faculty of Medicine and Health Sciences, Stellenbosch University (ref. N12/10/065) and from the Institutional Review Board of The Pennsylvania State University (ref. 41940). Written informed consent was obtained from all participants.

The 104 participants were assessed for eligibility, and 100 HIV-1–uninfected individuals (Xhosa, $n = 50$; Cape Mixed, $n = 50$) were enrolled. The summer and winter visits began 6 wk postsolstice. At the winter visit, all

participants received six capsules of 50,000 IU cholecalciferol (D3-50; Biotech Pharmaceutical), to be taken weekly, and Xhosa participants were followed up 6 wk (± 15 d; mean +3 d) after their winter visit (Fig. S1). Previous studies have shown that serum 25(OH)D plateaus after ∼4–6 wk of supplementation (29); however, it is possible that the final equilibrated 25(OH)D level was underestimated at 6 wk. Ethics, recruitment, follow-up, and exclusion criteria are detailed in *SI Materials and Methods*. The procedures described below were conducted at each study visit.

UVB Exposure. Sun exposure was assessed at each visit by a retrospective questionnaire that captured duration and time of day of outdoor sun exposure and the amount of skin not covered by clothing or a hat at time of exposure. PNUVB was calculated as described in *SI Materials and Methods*. Direct measurements of UVB were made at the University of Stellenbosch Solar Resource and Weather Station.

Skin Reflectometry. Skin reflectance of all research subjects, expressed as EI and MI, was measured using a portable reflectometry device (DSM II ColorMeter; Cortex Technology). Constitutive pigmentation was measured on the upper inner arm site, and facultative pigmentation was measured on the forehead. Three independent measures were taken from each site, and the mean was calculated.

Food Questionnaire. Intake of vitamin D (vitamin D_2 and vitamin D_3) was estimated using a 7-d quantitative food frequency questionnaire administered to every participant by a trained researcher at each study visit. The questionnaire (described in *SI Materials and Methods*) was adapted from a food frequency questionnaire shown to be valid in providing a reasonable estimation of dietary vitamin D intake in healthy young adults of diverse ancestry (30). Individuals taking vitamin D supplements were excluded.

Biochemistry and FBC. Peripheral blood collected in serum tubes was analyzed on the day of collection for 25(OH)D concentration by the chemiluminescent LIAISON 25 OH Vitamin D TOTAL Assay (DiaSorin). Serum also was stored at −80 °C and subsequently batch analyzed for DBP, acute phase markers, calcium, and albumin. FBCs were conducted within 2–3 h of collection. Assays are described in *SI Materials and Methods*.

SNP Genotyping. DNA was extracted from PBMCs using the QIAmp DNA Blood Mini Kit (QIAGEN), and 10 ng was analyzed by TaqMan Genotyping assay (Life Technologies), in duplicate, for the following SNPs in genes: *DBP* (rs7041 and rs4588), *DHCR7* (rs12785878), *CYP2R1* (rs10741657), *CYP24A1* (rs6013897), *CYP27B1* (rs10877012), and *VDR* (rs2544037, rs10783219, rs10735810, and rs731236), as described in *SI Materials and Methods*.

PBMC HIV-1 Infection and p24 Analysis. After the isolation of PBMCs, cells were infected immediately with HIV-1, as described in *SI Materials and Methods*. HIV-1 replication was measured by quantifying the HIV-1 p24 antigen concentration of lysed supernatant by Luminex, as described in ref. 31, with slight modifications (*SI Materials and Methods*).

Statistical Analysis. All univariate statistics were conducted in GraphPad Prism 6.0 software with an alpha of 0.05 and two-sided testing. GLM was conducted using Qlucore Omics Explorer 2.2 software and stepwise regression, and GLM on vitamin D optimality and deficiency was conducted in S-Plus. Full details are given in *SI Materials and Methods*.

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