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Changes in bone turnover markers with HIV seroconversion and ART initiation

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Background: Osteoporosis is common among HIV-infected persons and contributes to risk of fragility fracture. While ART initiation is associated with decreases in bone mineral density and increases in bone turnover, the impact of HIV on bone metabolism is unclear.

Methods: We identified men at the Chicago site of the Multicenter AIDS Cohort Study who HIV seroconverted while under observation. Concentrations of 25-OH vitamin D, bone turnover markers [procollagen type 1 N terminal propeptide (P1NP), osteocalcin (OC), C-telopeptide (CTX)] and sclerostin were measured from stored serum obtained at pre-HIV infection, pre-ART and post-ART initiation timepoints. Mixed models, with each biomarker as an outcome, were fitted. Timepoint, age, CD4 count (cells/mm^3), HIV-viral suppression, season and an age by timepoint interaction term were considered as fixed effects.

Results: Data from 52 participants revealed that median duration between HIV seroconversion and ART initiation was 8.7 years (IQR 3.7–11.6). Median CD4 and plasma HIV-RNA concentrations were 445 (IQR 298.5–689) and 20 184 copies/mL (IQR 6237–64 340), respectively, at the pre-ART timepoint. Multivariate analyses demonstrated pre-HIV infection levels of OC that were higher than pre-ART levels (6.8 versus 5.7 ng/mL, $P = 0.04$); and pre-ART levels of sclerostin that were higher than post-ART levels (0.033 versus 0.02 ng/mL, $P < 0.001$). No changes in P1NP, CTX and 25-OH vitamin D levels were detected.

Conclusions: HIV seroconversion was associated with decreased OC levels while ART initiation was associated with decreases in sclerostin, a negative regulator of bone formation. Our results suggest that both HIV infection and ART have an impact on bone metabolism in white men.

Introduction

Osteoporosis, which is characterized by a decrease in bone mineral density (BMD) and bone quality, is more common among HIV-infected compared with HIV-uninfected persons[1] and accounts in part for the higher than expected risk of fracture in HIV-infected populations.[2–4] Multiple studies have shown that initiation of ART is associated with decreases in BMD and increases in bone turnover biomarkers in the first 96 weeks of treatment, regardless of the ART regimen used.[5–9] However, the impact of HIV infection itself on bone metabolism is unclear.

Bone is constantly remodelling, with older bone being resorbed by osteoclasts and new bone being formed in its place by osteoblasts. Osteoblast function is reflected by markers of bone formation, including osteocalcin (OC, a bone matrix protein) and a procollagen type 1 N terminal propeptide (P1NP). Osteoclast function can be measured by an increase in serum levels of C-telopeptide (CTX) which is a degradation product of type 1 collagen. These biomarkers are recommended by the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine as reference analyses for bone turnover markers in clinical studies.[10]

More recently, sclerostin, a glycoprotein produced by osteocytes, was identified as a circulating factor which down-regulates bone formation.[11,12] In the general population, lower sclerostin levels have been associated with higher bone fracture risk, lower BMD and higher bone turnover rate.[13] These lower levels of sclerostin are thought to represent a compensatory mechanism to promote bone formation in the event of bone loss.[14] To our

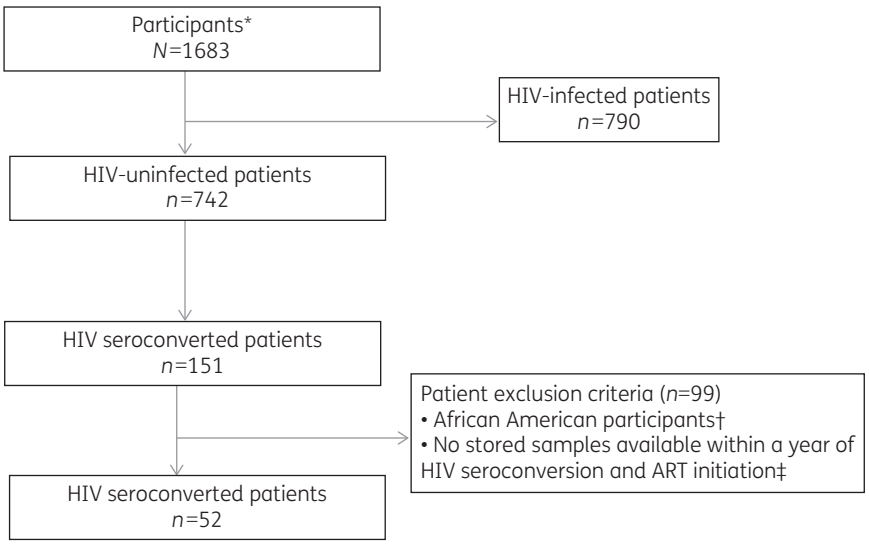

Figure 1. MACS cohort flow chart (1984–2013) at the Chicago site in 2013. *The MACS cohort included MSM at risk for HIV infection. †African Americans were excluded because of reported racial differences in level of 25-OH vitamin D. ‡6 months before or after HIV seroconversion and 6 months before or after ART initiation.

knowledge, no studies to date have investigated the effect of HIV seroconversion or ART initiation on sclerostin levels.

In addition, vitamin D deficiency (25-OH vitamin D <10 ng/mL) is common in the general population[15] and is associated with osteomalacia, osteopenia and osteoporosis.[16,17] Vitamin D deficiency among HIV-infected persons receiving ART has been described and data indicate relationships between 25-OH vitamin D deficiency, low CD4 and use of specific ART drugs such as efavirenz.[7,17,18] It is not known, however, whether HIV seroconversion affects 25-OH vitamin D concentrations. A recent analysis conducted in a large European cohort suggested that vitamin D deficiency is associated with immune dysfunction or increased inflammation.[19]

To address these knowledge gaps, we undertook a longitudinal, nested cohort study within the Multicenter AIDS Cohort Study (MACS) to assess associations between HIV seroconversion, ART initiation, levels of bone turnover regulation markers and 25-OH vitamin D.

Methods

Study population

The MACS is a prospective study of MSM who are HIV-infected or at risk for HIV-1 infection and has been ongoing since 1984 at four sites in the United States. Men attend semi-annual visits that include physical examinations and blood collection for laboratory testing and storage.

Ethics

Details of the study design and methods have been published.[20] The institutional review boards of each site approved the study protocols and informed consent was obtained from each participant. The approval reference number for this study is STU00060482-CR0002.

Selection criteria

Over 29 years, 1683 HIV-infected and HIV-uninfected men have been enrolled at the MACS Chicago site. Of these, 151 out of 893 HIV-uninfected men HIV seroconverted while under observation. We selected HIV-seroconverters with study visits before and after seroconversion, and before and after ART initiation (Figure 1). Bone turnover markers (P1NP, OC, CTX), sclerostin and 25-OH vitamin D were measured from serum obtained in the pre-HIV, pre-ART and post-ART periods. Participants were selected based on availability of stored samples from at least two of the three timepoints of interest closest to the event of interest (pre-HIV, pre-ART and post-ART). We excluded African American men from our study because of racial differences in 25-OH vitamin D levels and bone metabolism.[21]

Laboratory methods

Serum samples were stored at −80°C until analysis without prior thawing. All bone turnover biomarker measurements were centralized using frozen serum samples and performed at Johns Hopkins Bayview Advanced Chemistry Laboratory, Baltimore, MD, USA. CTX was measured using an enzyme-immunosorbent assay (Osteometer BioTech). OC was measured using immune-radiometric assay (Nichols Institute Diagnostics). Median intra-assay coefficients of variations were 8.2% and 3.0%, respectively, and the median inter-assay coefficients of variation were 0.01% and 6.3%, respectively. The expected normal concentration range was 3.4–11.7 ng/mL for OC and 0.115–0.748 ng/mL for CTX. P1NP was measured using immune-radiometric assay (IDS, Inc.), Sclerostin was measured by MSD assay (median inter-assay coefficient of variation 10.9%; the median intra-assay coefficient of variation was 2.4%) and 25-OH vitamin D by LC-MS/MS (Quest Diagnosis).[22]

Statistical analysis

Preliminary comparisons of bone markers and 25-OH vitamin D levels were made in paired samples for the following: pre-HIV infection versus pre-ART initiation, pre-ART initiation versus post-ART initiation and pre-HIV infection versus post-ART initiation. *P* values were obtained using Wilcoxon signed rank tests.

Multivariate analyses were performed using levels of each bone marker and 25-OH vitamin D as outcome variables in separate mixed models. To obtain parsimonious models that provided good fit, full models were specified and evaluated. Variables of interest included clinical

Table 1. Characteristics of the 52 HIV seroconverters

Characteristic	Value
Age at seroconversion (years), median (IQR)	35.3 (31.1–42.9)
Caucasians (%)	94.6
BMI at seroconversion (kg/m^2), median (IQR)	24.5 (21.4–26.6)
CD4 T cell count at ART initiation (cells/mm^3), median (IQR)	445 (298.5, 689)
HIV-RNA at ART initiation (copies/mL), median (IQR)	20 184 (6237–64 340)
Period of ART initiation, *n* (%)	
prior to 2003	27 (51.9)
2003–06	7 (13.5)
2007 or later	18 (34.6)
TDF-based ART regimen, *n* (%)	4 (7.7)
PI-based ART regimen, *n* (%)	21 (40.4)

TDF, tenofovir disoproxil fumarate.

timepoint (pre-HIV, pre-ART, post-ART), age (centered at 42.7 years), CD4 at ART initiation, viral suppression after ART initiation (yes/no), season [spring/summer (1 April–30 September) versus autumn/winter (1 October–31 March)], and a clinical timepoint by age interaction term. Final models included clinical timepoint and any fixed variables of interest determined to be significant. Models included a random intercept; observations were clustered by subject. All analyses were performed using SAS version 9.4.

Results

Baseline characteristics

Between 1984 and 2013, 52 men who HIV seroconverted who had at least two samples available were selected. Of those, 10 participants (19.2%) had available samples for all three time periods (pre-HIV/pre-ART/post-ART), 18 (34.6%) for the pre-HIV/pre-ART periods, 15 (28.9%) for the pre-HIV/post-ART periods and 9 (17.3%) for the pre-ART/post-ART periods for a total of 114 samples (37.7% pre-HIV, 32.5% pre-ART and 29. 9% post-ART samples). Among the 114 samples collected, 62 (54%) were collected in spring/summer and 52 (46%) were collected in autumn/winter. The median time (IQR) between the pre-HIV sample and HIV seroconversion was 2.7 years (IQR 1.2–7.1), median time between seroconversion and pre-ART samples was 1.5 years (IQR 0.1–2.9), median time between pre-ART samples and ART initiation was 9.4 years (IQR 2.5–11.4) and the median time between ART initiation and post ART samples was 5 years (IQR 2.1–7.5). Overall, the median time between HIV infection and ART initiation was 8.7 years (IQR 3.7–11.6).

Characteristics of patients who seroconverted are presented in Table 1. Participants were mostly Caucasian (94.6%), with a normal body mass index (median 24.5 kg/m^2) and a median age of 35.3 years at seroconversion. At the pre-ART visit, the median CD4 was 445 cells/mm^3 (IQR 298.5–689) and the median HIV-RNA level was 20 184 copies/mL (IQR 6237–64 340). Most participants initiated ART prior to 2003 and a few ($n = 4$; 7.7%) had received a tenofovir disoproxil fumarate (TDF)-based ART regimen. At the post-ART visit, the median (IQR) CD4 cell count was 422.5/mm^3 (IQR 332–641) and 20 (59%) had HIV-RNA below the limit of detection.

A univariate comparison of bone marker levels at the pre-HIV/pre-ART and pre-ART/post-ART periods is presented in Tables 2 and 3. Median OC levels prior to HIV seroconversion were higher than at the post-seroconversion/pre-ART timepoint [7.02 ng/mL (IQR 4.97–8.20) versus 4.60 ng/mL (IQR 3.49–7.18) ($P = 0.04$)]. While median P1NP, CTX and 25-OH vitamin D levels were relatively unchanged by seroconversion, estimated medians tended to be lower at the post-seroconversion/pre-ART timepoint. Median sclerostin levels were unchanged upon seroconversion ($P = 0.93$).

Comparisons between the pre- and post-ART periods revealed no difference in median CTX, P1NP, OC and 25-OH vitamin D levels. Median pre-ART sclerostin levels were higher than at the post-ART timepoint [0.030 ng/mL (IQR 0.021–0.047) versus 0.021 ng/mL (IQR 0.016–0.031) ($P = 0.02$)]; median sclerostin levels before HIV seroconversion were higher than at the post-ART timepoint (0.039 versus 0.018 ng/mL, $P < 0.001$).

The results of the multivariate analyses are presented in Table 4. The final models for each outcome included HIV timepoint and any fixed covariate that was found to be significant in the full model. For the model fitting, CTX, HIV timepoint and CD4 count were included as part of the multivariate analysis. For 25-OH vitamin D, HIV timepoint and season were included as part of the multivariate analysis. For P1NP, HIV timepoint and age (centred) were included. For OC and sclerostin, HIV timepoint was the only effect that merited inclusion in the final model.

Pre-HIV levels of OC were higher than both pre-ART and post-ART levels [least square (LS) means 6.8 versus 5.7 ng/mL ($P = 0.04$) and 5.3 ng/mL ($P < 0.01$) at the pre-ART and post-ART period, respectively]. However, no differences between pre-ART and post-ART OC levels were detected ($P = 0.47$). There was no significant association between HIV timepoints and CTX ($P = 0.20$) or P1NP ($P = 0.86$). Finally, we confirmed our univariate findings regarding the association between ART initiation and decreases in sclerostin levels (LS means 0.033 versus 0.020, $P < 0.001$). Median sclerostin levels prior to HIV seroconversion were higher than at the post-ART timepoint (LS means 0.036 versus 0.020 ng/mL, $P < 0.001$). As expected, 25-OH vitamin D levels adjusted for season were lower during autumn/winter compared with spring/summer (P value < 0.001) but no significant association between HIV timepoints and 25-OH vitamin D levels was apparent ($P = 0.14$).

Discussion

To our knowledge, this is the first study to provide evidence that HIV infection itself, independent of ART, affects bone turnover. Using a longitudinal design, we found that HIV seroconversion was associated with lower levels of OC, a key marker of osteoblast activity. We also found that ART initiation was associated with lower sclerostin levels, suggesting that ART initiation may affect osteoblast regulation. Taken together, our findings suggest that both HIV infection and ART impact bone metabolism.

Low BMD in HIV-infected patients is thought to be multifactorial in aetiology. HIV infection has been postulated to contribute to low BMD through the direct effects of both pro-inflammatory cytokines and HIV viral proteins, which have been shown to increase osteoclast activity and impair osteoblast function *in vitro*.[23,24]

Table 2. Univariate analysis pre-HIV/pre-ART comparisons of bone biomarker levels

Parameter	Median	IQR	*P* value[a]
CTX[b] (ng/mL)			0.31
pre-HIV	0.300	0.235–0.425	
pre-ART	0.265	0.160–0.380	
P1NP[c] (ng/mL)			0.21
pre-HIV	47.37	37.68–60.73	
pre-ART	43.40	33.58–56.27	
OC[d] (ng/mL)			0.04
pre-HIV	7.02	4.97–8.20	
pre-ART	4.60	3.49–7.18	
Sclerostin (ng/mL)			0.93
pre-HIV	0.031	0.026–0.041	
pre-ART	0.031	0.021–0.039	
25-OH vitamin D (ng/mL)			0.06
pre-HIV	36.58	29.23–45.46	
pre-ART	33.10	25.53–39.52	

[a]*P* values obtained using Wilcoxon signed rank tests. Tests compared pre-HIV samples with paired pre-ART samples ($n = 28$), pre-ART samples with paired post-ART samples ($n = 19$) and pre-HIV samples with paired post-ART samples ($n = 25$).
[b]CTX, C-telopeptide.
[c]P1NP, procollagen type-1N terminal propeptide.
[d]OC, osteocalcin.

Table 3. Univariate analysis pre-ART/post-ART comparisons of bone biomarker levels

Parameter	Median	IQR	*P* value[a]
CTX[b] (ng/mL)			0.79
pre-ART	0.330	0.20–0.48	
post-ART	0.320	0.23–0.44	
P1NP[c] (ng/mL)			0.31
pre-ART	48.54	33.9–65.9	
post-ART	44.01	35.9–56.2	
OC[d] (ng/mL)			0.46
pre-ART	6.50	3.88–7.90	
post-ART	5.64	4.13–6.73	
Sclerostin (ng/mL)			0.02
pre-ART	0.030	0.021–0.047	
post-ART	0.021	0.016–0.031	
25-OH vitamin D (ng/mL)			0.80
pre-ART	30.74	19.48–36.88	
post-ART	28.67	21.21–34.42	

[a]*P* values obtained using Wilcoxon signed rank tests. Tests compared pre-HIV samples with paired pre-ART samples ($n = 28$), pre-ART samples with paired post-ART samples ($n = 19$) and pre-HIV samples with paired post-ART samples ($n = 25$).
[b]CTX, C-telopeptide.
[c]P1NP, procollagen type-1N terminal propeptide.
[d]OC, osteocalcin.

A transgenic rat model of HIV infection described by Vikulina *et al.*[24] demonstrated profound disturbances in bone metabolism with significantly increased levels of a marker of bone resorption (RANKL) and histologically increased numbers of osteoclasts compared to WT rats, while there were no significant changes in levels of bone formation biomarkers.

By examining the same men before and after HIV seroconversion, our study has taken a unique approach to evaluating whether HIV infection itself is associated with changes in bone metabolism. In a previous study,[22] we showed an uncoupling of bone formation and resorption among HIV-infected persons, demonstrating that while 34.5% had CTX levels above the normal range before ART initiation, OC was relatively suppressed. Similarly, in the present study, we found lower OC levels after seroconversion. We did not see any effects of HIV seroconversion on other markers of bone formation (P1NP) or bone resorption (CTX). However, this could be explained by the small sample size used for our study.

ART initiation also contributes to osteoporosis and fracture risk. In multiple studies, ART initiation has been associated with a rapid increase in levels of both markers of bone formation and bone resorption.[6,25,26] These changes are seen as early as 2 weeks post ART initiation, plateau at 96 weeks and are associated with bone loss.[22,25,26] In our study, we found no effect on levels of markers of bone turnover between the pre-ART and the post-ART sample. There could be several explanations for this. First, only 4 out of our 52 studied men who seroconverted were treated with a TDF-containing regimen, which may have limited the impact of ART initiation on bone metabolism. Second, our population initiated ART at higher CD4 and lower HIV-RNA levels compared with participants in other studies.[25] ART initiation with lower CD4 counts and higher HIV-RNA levels has been associated with more profound decreases in BMD, independent of the ART regimen.[27] Next, ~60% of the men who initiated ART had undetectable HIV-RNA at the time of the post-ART sample. The majority of studies which have examined bone turnover with ART initiation have focused on rates of virological suppression of >80%. Although the relatively high prevalence of unsuppressed HIV-RNA with ART observed in our study was not unusual at that time (the majority initiated ART prior to 2003), the inclusion of men both with and without suppressed HIV-RNA may have affected our bone turnover marker results. Finally, the median time elapsed between studied samples collected after seroconversion and ART initiation was long (9.4 years, IQR 2.5–11.4) and may have decreased the apparent impact of ART initiation on bone turnover.

We found that sclerostin levels were lower in the post-ART period compared to pre-ART levels. These findings complement findings from other studies conducted in HIV-infected populations: in a cross-sectional study of ART-treated individuals, lower plasma sclerostin was associated with lower spine BMD, consistent with findings from the general population.[28] Similarly, improvements in BMD and reductions in bone turnover observed among TDF-treated persons who switched to abacavir-containing ART regimens were accompanied by an increase in circulating sclerostin levels.[29] As sclerostin has a major effect on osteoblasts through the Wnt/β-catenin pathway,[30] our findings suggest that ART initiation could affect osteoblast regulation and that the Wnt/β-catenin pathway may be an important target for intervention to prevent ART-associated bone loss.

Table 4. Multivariate analysis pre-HIV/pre-ART/post-ART comparisons of bone biomarker levels

Parameter	LS mean	SE	*P* value
CD4-adjusted CTX[a] (ng/mL)			timepoint a = b = c; 0.20
(a) pre-HIV	0.38	0.026	
(b) pre-ART	0.32	0.026	
(c) post-ART	0.34	0.026	
Age-adjusted P1NP[b] (ng/mL)			timepoint a = b = c; 0.86
(a) pre-HIV	48.7	2.78	
(b) pre-ART	47.1	2.60	
(c) post-ART	47.4	3.40	
OC[c] (ng/mL)			timepoint a = b = c; 0.01
(a) pre-HIV	6.8	0.40	a = b; 0.04
(b) pre-ART	5.7	0.50	
(c) post-ART	5.3	0.48	a = c; <0.01
Sclerostin (ng/mL)			timepoint a =b = c; <0.001
(a) pre-HIV	0.036	0.002	a = c; <0.001
(b) pre-ART	0.033	0.003	b = c; <0.001
(c) post-ART	0.020	0.002	
25-OH vitamin D (ng/mL) adjusted for season			timepoint a = b = c; 0.14
summer			
(a) pre-HIV	39.5	1.89	
(b) pre-ART	37.0	2.11	
(c) post-ART	35.2	2.35	
winter			
(a) pre-HIV	31.6	2.27	
(b) pre-ART	29.1	2.20	
(c) post-ART	27.2	2.09	

LS mean, least square mean.
[a]CTX, C-telopeptide.
[b]P1NP, procollagen type-1N terminal propeptide.
[c]OC, osteocalcin.

Our study had several limitations not mentioned previously. Time intervals were wide and variable especially between the pre-ART period and post-ART initiation. In addition, because of the small sample size, we were unable to make valid comparisons between specific ART regimens or medications in terms of their effects on bone markers. We used serum stored for an extended period. However, bone biomarkers levels have been shown to be stable after long-term storage at −80°C.[31] Finally, we cannot extrapolate our findings to other HIV-infected populations, including persons of African ancestry and women.

In conclusion, we demonstrated that both HIV infection and ART have an impact on bone metabolism. HIV seroconversion is associated with decreases in levels of a key bone formation biomarker and ART initiation with decreases in levels of sclerostin, a negative regulator of bone formation. Further studies are needed to better understand the implications of these findings, particularly among ageing HIV-infected persons.

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Multicenter AIDS cohort study

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L. S. reports personal fees from Gilead, ViiV, Janssen Pharmaceuticals, AIDS International Education project, outside the submitted work. S. R. reports no conflict of interest. J. P. reports grant from the MACS and personal fees from Pfizer. F. J. P. reports personal fees from Gilead Sciences, Janssen Pharmaceuticals, Merck and Co. and Bristol Myers Squibb outside the submitted work. T. T. B. reports personal fees from Gilead Sciences, Merck and Co., Bristol Myers Squibb, EMD-Serono, Theratechnologies, Abbvie outside the submitted work.

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