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Effects of HIV infection and antiretroviral therapy with ritonavir on induction of osteoclast-like cells in postmenopausal women

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Abstract

Summary—Ritonavir (RTV) is a commonly used antiretroviral associated with bone loss. We show that peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus (HIV)-positive women on RTV are more likely to differentiate into osteoclast-like cells when cultured with their own sera than PBMCs and sera from HIV[−] women or HIV⁺ on other antiretrovirals.

Introduction—RTV increases differentiation of human adherent PBMCs to functional osteoclasts in vitro, and antiretroviral regimens containing RTV have been associated with low bone mineral density (BMD) and bone loss.

Methods—BMD, proresorptive cytokines, bone turnover markers (BTMs), and induction of osteoclast-like cells from adherent PBMCs incubated either with macrophage colony-stimulating factor (MCSF) and receptor activator of nuclear factor κ B ligand (RANKL) or with autologous serum were compared in 51 HIV[−] and 68 HIV⁺ postmenopausal women.

Results—BMD was lower, and serum proresorptive cytokines and BTMs were higher in HIV⁺ versus HIV[−] women. Differentiation of osteoclast-like cells from adherent PBMCs exposed to either MCSF/RANKL or autologous serum was greater in HIV⁺ women. Induction of osteoclast-like cells was greater from PBMCs exposed to autologous sera from HIV⁺ women on RTV-containing versus other regimens ($172 \pm 14\%$ versus $110 \pm 10\%$, $p < 0.001$). Serum-based induction of osteoclast-like cells from adherent PBMCs correlated with certain BTMs but not BMD.

Conclusions—HIV infection and antiretroviral therapy are associated with higher BTMs and increased differentiation of osteoclast-like cells from adherent PBMCs, especially in women on regimens containing RTV. HIV⁺ postmenopausal women receiving RTV may be at greater risk for bone loss.

Keywords

Bone; HIV; Osteoclast; Postmenopausal; Protease inhibitor

Introduction

Bone mineral density (BMD) is commonly reported to be lower in patients with human immunodeficiency virus (HIV) infection than in HIV[−] controls [1]. The etiology of bone loss in HIV-infected patients is multifactorial. Known risk factors for osteoporosis, such as low body weight [2] and hypogonadism [3, 4], are common in HIV⁺ patients. HIV may also have direct effects on osteoclasts and osteoblasts [5, 6]. In addition, certain cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) which are elevated in HIV infection may increase osteoclast activation by induction of receptor activator of nuclear factor κ B ligand (RANKL), the primary cytokine responsible for osteoclast differentiation and bone resorption [7].

Several studies implicate antiretroviral therapy (ART) [8–11] in the pathogenesis of low BMD, while others have not confirmed this association [12–16]. However, as antiretroviral drugs from the same class may affect bone cells very differently, inter- and intraclass drug differences likely confound these associations [17–19]. Ritonavir (RTV) is a protease inhibitor (PI) that is widely utilized in low doses to “boost” serum concentrations of other PIs such as lopinavir, fosamprenavir, atazanavir, and darunavir. Initiation of RTV-boosted PI regimens (RTV-PI) is associated with greater bone loss than PI regimens without RTV [16], triple nucleoside reverse transcriptase inhibitor (NRTI) regimens [20], and non-NRTI (NNRTI)-based regimens [21].

Osteoclasts, the primary cells responsible for bone resorption, are multinucleated cells that differentiate from circulating mononuclear cell precursors of monocyte/macrophage lineage [22, 23]. Macrophage colony-stimulating factor (MCSF) and RANKL are required for osteoclast differentiation. We previously reported induction of RANKL in cultures of CD4⁺ T lymphocytes exposed to HIV-1 envelope glycoprotein gp120 at concentrations present in plasma of untreated HIV⁺ individuals [17, 24]. Exposure to supernatants from such cultures increased differentiation of adherent peripheral blood mononuclear cells (PBMCs) into

functional osteoclasts [17]. This observation could explain the link between untreated HIV infection and low BMD, as serum RANKL levels correlate inversely with BMD in HIV+ individuals [25]. We also reported that RTV, at concentrations present in patients on typical RTV-PI regimens, increased osteoclast differentiation from human and murine adherent PBMCs exposed to RANKL plus MCSF or to supernatants from PBMCs cultured with HIV-1 envelope glycoprotein gp120, while PIs with no reported link to bone loss, including nelfinavir and indinavir, did not [17].

In a recent prospective cohort study of HIV+ postmenopausal minority women, we reported that HIV+ women have lower BMD and higher bone turnover markers (BTMs) and serum TNF- α than HIV- women and that HIV status was independently associated with low BMD after controlling for age, race/ethnicity, and BMI [26]. When TNF- α or the resorption marker, serum N-telopeptide (NTx), was forced into the model, the association between HIV status and BMD was lost, providing evidence that lower BMD in HIV+ postmenopausal women is mediated by increased bone resorption, possibly due to high levels of proresorptive cytokines [26]. Given these results, our in vitro data [17, 24], and evidence of bone loss with initiation of RTV-PI-based regimens reported by others [20, 21], we hypothesized that HIV+ women, particularly those on RTV-PI regimens, would have in vitro evidence of increased osteoclast differentiation. We utilized an in vitro assay of osteoclast induction from adherent PBMCs to test this hypothesis in a subset of our cohort of postmenopausal HIV+ women.

Methods

Subjects

One hundred and eighty-seven (95 HIV- and 92 HIV+) minority postmenopausal women were recruited into a longitudinal study to assess the determinants and etiology of low BMD and bone loss from Columbia University Medical Center (CUMC) and Bronx-Lebanon Hospital Center (BLHC) in New York; the baseline evaluation was reported recently [26]. This is a substudy of the parent cohort study that includes 119 subjects (51 HIV- and 68 HIV+) with PBMCs and sera obtained on the same day, which was available for the in vitro osteoclast assay. All subjects in the parent cohort were over 40, Hispanic or African American, and postmenopausal as defined by ≥ 1 year of amenorrhea; in addition to amenorrhea, serum follicle stimulating hormone (FSH) had to be >30 or >20 mIU/ml with an estradiol <30 pg/ml or age >55 , regardless of estradiol and FSH levels. Exclusion criteria included metabolic bone disease, multiple myeloma, solid tumors with bone metastases, endocrinopathies, serum creatinine >1.5 mg/dl, celiac or inflammatory bowel disease, current glucocorticoid or anticonvulsant use, and current or past treatment of osteoporosis. In addition, women on current hormone replacement therapy were excluded from these analyses because of the effects of estrogen to inhibit osteoclast-mediated bone resorption.

Medical, surgical, and reproductive history, osteoporosis risk factors, current and past medication history, and HIV and ART history were obtained by interview and chart review. Years since menopause was calculated as time since 1 year after last menstrual period. In hysterectomized women, years since menopause was calculated as time since 1 year after onset of menopausal symptoms or set at age 50 if no menopausal symptoms were recalled. Control subjects met the same criteria; enzyme-linked immunosorbent assay (ELISA) testing verified HIV- status.

All subjects gave written informed consent. The Institutional Review Boards of CUMC, BLHC, and Weill Cornell Medical Centers (WCMC) approved the study.

Bone mineral density (BMD)

BMD of the lumbar spine (LS; L1–4), femoral neck (FN), total hip (TH), and nondominant one-third radius (DR) was measured at CUMC by dual energy X-ray absorptiometry utilizing a QDR 4500 bone densitometer. Instruments were calibrated using reference spine and hip phantoms with anatomically correct contours (Hologic, Inc.). Reproducibility with the spine phantom is 0.51% ($n=178$), and short-term in vivo reproducibility (coefficient of variation) is 0.68% for the LS, 1.36% for the TH, and 0.70% for the DR.

Clinical laboratory methods

Fasting morning serum was processed immediately, stored in aliquots at -80°C , and batch-analyzed in research laboratories at CUMC for parathyroid hormone (PTH; RIA, Corning-Nichols Laboratory, San Clemente, CA, USA; inter-assay precision is 8.3%; sensitivity is 1 pg/ml), serum 25-hydroxyvitamin D (25-OHD; Diasorin RIA, Stillwater, MN, USA; inter-assay precision is 15%; sensitivity is 2 ng/ml), serum 1,25-dihydroxyvitamin D (Diasorin, Stillwater, MN, USA; inter-assay precision is 15%; sensitivity is 2 pg/ml), and estrone (RIA, Diagnostic Systems Laboratories, Inc., Webster, TX, USA; inter-assay precision is 9.3%; sensitivity is 1.2 pg/ml). Formation markers included bone-specific alkaline phosphatase (BAP; Metra BAP, Quidel Corp., San Diego, CA, USA; inter-assay precision is 8%; sensitivity is 0.7 U/L) and osteocalcin (RIA, Immutopics, San Clemente, CA, USA; inter-assay precision is 12.1%; sensitivity is 0.05 ng/ml). Resorption markers included cross-linked N-telopeptide of type I bone collagen (NTx) by competitive-inhibition ELISA (Inverness Medical, Princeton, NJ, USA; inter-assay sensitivity is 11.4%; sensitivity is 5 nmol/BCE/L), C-telopeptide of type 1 collagen (CTx) by sandwich ELISA (IDS Ltd., Scottsdale, AZ, USA; inter-assay precision is 11%; sensitivity is 0.020 ng/ml), and tartrate-resistant acid phosphatase isoform 5b (TRAP5b) by ELISA (BoneTRAP, IDS Ltd., Scottsdale, AZ, USA; inter-assay precision is 7%; sensitivity is <1 U/L). TNF- α by ELISA (R&D Systems, Inc., Minneapolis, MN, USA; inter-assay precision is 17%; sensitivity is 0.12 pg/ml), IL-6 by ELISA (R&D Systems, Minneapolis, MN, USA; inter-assay precision is 10%; sensitivity is 0.04 pg/ml), and RANKL and osteoprotegerin (OPG) by ELISA were developed by our laboratory at WCMC [17] (inter-assay CV is 12%; and sensitivity is 40 pg/ml).

CD4 counts were measured by flow cytometry. HIV-1 RNA was quantified by the Amplicor HIV-1 Monitor Ultrasensitive Test (version 1.5) with a linear range of 50–100,000 copies/ml (Roche Diagnostics, Indianapolis, IN, USA).

Osteoclast induction assays

(a) RANKL-mediated osteoclast induction (ROI) assay—A variation of this assay of osteoclast differentiation [27] was reported previously by our laboratory [17]. HIV+ or HIV− donor PBMCs adherent to polystyrene following a 2-h incubation in RPMI 1640 at 37°C were collected using a tissue scraper. Adherent cells, 2×10^5 , were cultured in 0.5 ml RPMI 1640 plus RANKL (25 ng/ml) and MCSF (100 ng/ml; Peprotech, Rocky Hill, NJ, USA) and 10% fetal bovine serum (FBS) in 8-well glass slide Lab-Tek chambers (Nalge Nunc, Naperville, IL, USA) for 7 days. On day 4, half the culture medium was removed and replaced with fresh cytokines and FBS. Osteoclast-like cells were identified by two methods: direct visualization of staining for TRAP in multinucleated giant cells (≥ 5 nuclei/cell) and quantitation of TRAP enzyme activity (Sigma kit, St. Louis, MO, USA), expressed as optical density (OD) units at 405 μm absorbance. The possibility that TRAP+ inflammatory cells such as neutrophils could be confused with mature osteoclasts was excluded by TRAP staining of PBMCs on culture initiation; no TRAP+ cells were seen. In previous studies by our group, the authenticity of these osteoclasts was confirmed for

randomly selected samples representing HIV– and HIV+ groups using direct bone resorption assays with osteologic discs and dentine slices [17].

(b) Serum-based autologous osteoclast induction assay (AOI)—In a pilot experiment, we assessed osteoclast differentiation from 2×10^5 adherent cells derived from PBMCs from both HIV– and HIV+ individuals cultured for 7 days only in 0.2-ml RPMI 1640 containing varying concentrations (1–25%) of autologous serum. On day 4, half the medium was removed and replaced with fresh RPMI 1640 plus autologous serum. A plateau in terms of maximal number of osteoclast-like cells formed, quantitated by TRAP activity and enumeration of TRAP+ multinucleated cells, was seen at 15% serum (v/v). This concentration was employed in all studies reported here. Values are presented as percent increase in TRAP activity over background, which corrects for differences in background OD in different assay runs.

Statistical analysis

Distributions of continuous variables were evaluated with Kolmogorov–Smirnov tests, and all measure failing criteria for normality were log transformed prior to statistical analysis (raw scores are reported). Group differences between HIV+ and HIV– demographic and clinical characteristics were evaluated with Student's *t* test for continuous variables and Chi-square or Fisher's exact test for categorical variables without adjustment for multiple comparisons. Statistical estimates of subgroup differences between HIV– and HIV+, between HIV+/ART– and HIV+/ART+, and between HIV+ ART+/RTV– and ART+/RTV+ in calciotropic and gonadal hormone levels (four variables), proresorptive cytokines (four variables), and BTM levels (five variables) used Bonferroni-adjusted criterion *p* values to control for multiple comparisons within each set of variables.

Multiple regression analysis was used to evaluate BMD differences by HIV status after adjusting for age, race/ethnicity, and BMI. Pearson correlation was used to estimate the strength of association between indices of osteoclast induction and proresorptive cytokines, BTMs, and BMD. Data are expressed mean±SEM.

Results

Characteristics of the study population

HIV+ women were younger, had fewer years since menopause, weighed less, and had less body fat than the HIV– women (Table 1). More HIV+ women were hepatitis C seropositive and reported alcohol use, but fewer reported hyperlipidemia or statin use. Absolute BMD and T-scores were lower in the HIV+ women at the LS, TH, and FN (Table 1). These results do not differ significantly from the previously reported data from the parent cohort (data not shown) [26]. In a multivariate model, HIV status remained a significant predictor of LS BMD after adjustment for known predictors of bone mass ($\beta=0.073$, $p<0.01$), including age, race/ethnicity, and BMI (total model adjusted $R^2=0.327$, $p<0.0001$). HIV+ women on ART had lower mean $\log_{10}$ HIV-1 RNA levels (4.88 ± 0.35 versus 7.61 ± 0.75 , $p<0.0001$) but similar CD4 counts than women not on ART.

Calciotropic and gonadal hormones, proresorptive cytokines, and BTMs

Serum biochemistries by HIV status are shown in Table 2. Within the HIV+ group, we also compared those on ART with those who were not; among those on ART, we compared HIV + ART+ women according to whether their ART regimen included RTV. Differences that remained significant after adjustment for multiple comparisons are presented in bold type. Serum estrone, the major circulating estrogen in postmenopausal women, and calciotropic hormones were similar between HIV+ and HIV– groups, as well as between ART groups

(Table 2). Bone resorption markers, NTx and CTx, were higher in HIV+ than HIV- women (Table 2), and CTx was higher in HIV+ women on ART than those who were not. TNF- α was higher in HIV+ than HIV- women but did not differ by ART status. OPG, measured by an assay that detects both free OPG and OPG bound to RANKL, was higher in HIV+ than HIV- women and in HIV+ women on ART (Table 2). OPG levels correlated with osteocalcin ($r=0.32$, $p<0.05$) and CTx ($r=0.45$, $p<0.01$).

There were no differences in serum PTH, 25-OHD, estrone, BTMs, and cytokine concentrations in treatment subgroups stratified by antiretroviral class: NRTI-only, NNRTI, or PI-based (data not shown). RTV-PI regimens included lopinavir ($N=10$), atazanavir ($N=7$), and fosamprenavir ($N=3$). The RTV-PI group had similar nadir CD4 counts (150 ± 31 vs. 182 ± 22 cells/mm³, $p=0.39$), median HIV-RNA levels, and time since HIV diagnosis as other ART groups. The ratio of NTx and CTx to TRAP5b, which has been purported to reflect the rate of resorption adjusted for osteoclast number [28], was slightly but not significantly higher in the RTV-PI group (NTx/TRAP5b, 9.8 ± 1.7 versus 5.2 ± 0.4 , $p<0.06$; CTx/TRAP5b, 0.26 ± 0.05 versus 0.18 ± 0.01 , $p<0.18$) than other ART groups.

Induction of osteoclast-like cells from PBMCs

To examine the effects of HIV infection and ART on induction of osteoclast-like cells from adherent PBMCs, we utilized two different in vitro models. The ROI assay examines the effects of maximal concentrations of RANKL and MCSF on adherent PBMCs from HIV+ and HIV- subjects; higher results likely reflect increased number or sensitivity of osteoclast precursors. The AOI assay, in which adherent PBMCs from HIV+ and HIV- subjects are cultured with their autologous sera, examines the effects of factors circulating in serum (e.g., cytokines, drugs, and viral proteins) on osteoclast precursors.

With the ROI assay, we observed higher levels of TRAP positivity in adherent PBMCs cultured from HIV+ than HIV- women (2.35 ± 0.08 versus 1.90 ± 0.13 OD; $p<0.01$, Fig. 1), indicating that exposure to high concentrations of MCSF and RANKL resulted in increased differentiation of osteoclast-like cells in HIV+ women. There were no significant differences in TRAP-positive osteoclast-like cells according to whether adherent PBMCs were from HIV+ women on or off ART, or in those on ART who were receiving RTV-boosted PI regimens (data not shown); however, the sample sizes of the subgroups were small for these comparisons.

With the AOI assay, we observed higher TRAP positivity after adherent PBMCs from HIV+ women than HIV- women were exposed to autologous sera ($129\pm 7\%$ versus $101\pm 8\%$; $p<0.02$; Fig. 2). There was no difference in TRAP positivity when PBMCs from HIV+ ART- and HIV+ ART+ women were compared ($132\pm 8\%$ versus $118\pm 19\%$). However, in the subset of HIV+ ART+ women receiving RTV, TRAP positivity was higher than in HIV+ ART+ women not receiving RTV ($172\pm 14\%$ versus $110\pm 10\%$, $p<0.001$).

Relationships between AOI and BTMs and BMD

In HIV+ women, there was a weakly positive association between AOI results and serum osteocalcin ($r=0.270$, $p<0.05$) and a modest to strong association with NTx/TRAP5b and CTx/TRAP5b indexes (Fig. 3), but not with any other BTM or cytokine. Exclusion of one subject who had the highest NTx/TRAP5b and CTx/TRAP5b index did not change the association between AOI and either index. Although serum TRAP5b was directly associated with NTx ($r=0.523$, $p<0.01$), CTx ($r=0.604$, $p<0.0001$), BAP ($r=0.384$, $p<0.05$), and osteocalcin ($r=0.395$, $p<0.05$), TRAP5b was inversely associated with the AOI results ($r=-0.435$, $p<0.01$).

Given the correlation between certain BTMs and AOI in HIV+ women, we examined whether BTMs and AOI results were associated with BMD. Among HIV+ women, there was a weak inverse association between osteocalcin and BMD at the TH ($r=-0.273$, $p<0.05$) and one-third radius ($r=-0.270$, $p<0.05$), but not other sites. Similarly, there was a weak inverse association between CTx ($r=-0.380$, $p<0.01$) and one-third radius BMD, but not other sites. Serum BAP and NTx were not associated with BMD at any site. AOI results were not associated with BMD at any site.

Discussion

The BMD and BTM results of this study reflect those of the parent study [26]; BMD is lower in HIV+ than HIV- postmenopausal women, and HIV status is a significant predictor of BMD after adjustment for known determinants of BMD. In terms of possible mechanisms for lower BMD in HIV+ women, serum proresorptive cytokines (serum TNF- α) and bone resorption markers (NTx and CTx) were higher in HIV+ women. New data reported in this study include serum RANKL levels, which were comparable between HIV+ and HIV- women, and serum levels of OPG, the decoy receptor for RANKL, which were markedly higher in HIV+ women. When exposed to maximal concentrations of exogenous RANKL, adherent PBMCs from HIV+ women exhibited greater induction of TRAP+ osteoclast-like cells than adherent PBMCs from HIV- women. When exposed only to 15% autologous serum, adherent PBMCs from HIV+ women also exhibited greater induction of osteoclast-like cells than adherent PBMCs of HIV- women. Adherent PBMCs exposed to autologous serum from HIV+ women receiving RTV-based ART demonstrated significantly higher induction of osteoclast-like cells than those on ART regimens that did not include RTV. However, though AOI assay results correlated with serum osteocalcin among HIV+ women, they did not correlate with other BTMs or with BMD.

ROI assays have demonstrated increased numbers of circulating and bone marrow-derived osteoclast precursors and/or greater sensitivity to high levels of RANKL/MCSF in two diseases characterized by increased bone resorption: Paget's disease of bone [29] and psoriatic arthritis [30]. In addition, large numbers of osteoclast precursors circulate in the blood of patients with multiple myeloma [31]. That we detected evidence of more circulating osteoclast-like cells after stimulation with RANKL and MCSF in HIV+ than HIV- postmenopausal women suggests that precursors are present in higher numbers and/or are more sensitive to the stimulatory effects of these cytokines. This observation is consistent with higher BTMs documented in the HIV+ subjects.

The AOI assay assesses whether HIV+ serum, which contains several factors not present in HIV- serum (e.g., antiretroviral drugs, HIV gene products, elevated cytokines, and others), stimulates differentiation of adherent PBMCs from the same individuals. That the AOI assay showed greater induction of osteoclast-like cells in HIV+ than HIV- women suggests that HIV+ women have increased numbers of osteoclast precursors and/or that these precursors have greater sensitivity to circulating factors. That the AOI results were significantly higher in women on RTV-PI than other ART regimens suggests that they have circulating osteoclast precursors that are either increased in numbers or more responsive to serum-based factors such as RTV. The AOI results are consistent with our in vitro findings that RTV, at concentrations present in patients on typical RTV-PI regimens, increased osteoclast differentiation from human and murine adherent PBMCs exposed to HIV-1 envelope glycoprotein gp120, while PIs with no reported link to bone loss, including nelfinavir and indinavir, did not [17, 24].

Many alterations in cell activation state and cytokine production persist in the setting of HIV infection, despite effective ART [32, 33]. Even when cytokine levels are lowered by ART,

certain antiretrovirals may facilitate their intracellular activity. This can occur via interference with processing of signaling intermediates and alterations in the Wnt/ β -catenin pathway, formerly thought to affect osteoclast differentiation only indirectly [34], as we demonstrated in vitro for RTV [24]. This may be the reason why we did not observe any difference in responsiveness to RANKL and MCSF with PBMCs from women receiving RTV using the ROI assay, in contrast to the AOI assay. It is possible that only the AOI assay, which utilizes both PBMCs and autologous sera, can differentiate between ART subgroups, since antiretrovirals present in the sera modify the response of PBMCs from HIV+ subjects [35, 36]. Alternatively, inconsistencies in the effects of RTV on induction of osteoclast-like cells between ROI and AOI assays may be due to an insufficient size for the ROI assay, since the number of ART+ subjects available for the ROI assays was smaller than that available for the AOI assay.

It is unclear that the AOI assay reflects what is transpiring in vivo. While the AOI assay differed between RTV-treated and untreated subjects, BTMs did not. Although the association between AOI and serum osteocalcin suggests that the assay reflects ongoing processes of bone remodeling, the association with osteocalcin was relatively weak, and no relationships between AOI and serum NTx or CTx were detected. Although NTx/TRAP5b and CTx/TRAP5b indexes have been interpreted as reflecting the rate of bone resorption adjusted for osteoclast number [28], they have been explored only in rodent models of osteopetrosis [37, 38], a disorder characterized in humans by marked discrepancies between osteoclast numbers (elevated) and osteoclast function (decreased) [38, 39]. Thus, while the relationships between the AOI results and the two indexes are significant, the clinical utility of such indexes has not been explored in human studies of osteoporosis or its therapy. In addition, the inverse association observed between the AOI assay and serum TRAP5b in HIV+ women is counterintuitive, particularly as TRAP5b correlated directly with all other BTMs. One possible interpretation could be that production and release of TRAP5b occur early in osteoclast differentiation, before fusion [38]. Thus, it is possible that if osteoclast precursors have already been stimulated to differentiate, serum TRAP5b is elevated, but precursors are less responsive to stimulatory effects of autologous sera.

Elevated BTMs were associated with lower BMD in both in the entire cohort and within the HIV+ women. However, we did not find statistically significant associations between AOI and BMD. The lack of correlation between AOI and BMD is not entirely unexpected. Osteoclast induction assays, like BTMs [40, 41], might be more likely to predict rates of bone loss over time. More information will become available regarding the relationships between AOI assays and bone loss upon completion of the longitudinal study. Our preliminary analyses found that AOI results were directly related to rates of bone loss at the one-third radius in HIV+ women [42]. The AOI assay is lengthy and cumbersome and unlikely to become a useful clinical test. However, it may prove helpful in unraveling the effects of various drugs on osteoclast biology in the setting of HIV infection. Future studies are planned with direct quantitation of osteoclasts in HIV+ subjects on various ART regimens.

The strengths of this study include its focus on postmenopausal women, a population classically at highest risk of bone loss and fractures, the inclusion of minority women, a rapidly growing segment of the HIV+ population, and enrollment of a clinic-based control population. Limitations include the modest-sized cohort, which provided sufficient power to evaluate cross-sectional differences in BMD by HIV status but not by ART class, and the availability of PBMCs and certain BTMs for only a subset of the study population. While our previous studies confirmed that the osteoclast-like cells resulting from the ROI and AOI assays excavated resorption pits on dentine slices [17], in this study, we identified osteoclasts by TRAP staining and did not prove that they are osteoclasts. Additionally, we

did not define osteoclast precursors by cell markers, nor were we able to validate these in vitro findings against the gold standard of bone histomorphometry. The osteoclast induction assays and cytokines may not reflect accurately the pathophysiology of the increased remodeling activity seen in HIV+ women. Additionally, our cross-sectional analysis precludes assessments of causal relationships between HIV status, ART exposure, AOI, and bone loss or fractures. Postmenopausal women constitute a minority of HIV-infected individuals, albeit a growing minority; the observations we have reported may not apply to younger women or to men.

In summary, we have confirmed that HIV+ women have lower BMD and higher BTMs and demonstrated that their adherent PBMCs exhibit evidence of greater induction of osteoclast-like cells than those of HIV- women, both when exposed to MCSF and RANKL or to autologous serum. Adherent PBMCs from HIV+ women receiving RTV-boosted PI regimens exhibit greater induction of osteoclast-like cells after exposure to autologous serum than PBMCs from women on regimens that do not contain RTV. These results raise concern that HIV+ subjects receiving RTV may be particularly susceptible to bone loss over long-term observation.

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Fig. 1.

Induction of osteoclast-like cells by exogenous receptor activator of nuclear factor κ B ligand (RANKL) and macrophage colony-stimulating factor (MCSF): the RANKL-mediated osteoclast induction assay. Peripheral blood adherent cells from HIV- ($N=11$) and HIV+ ($N=24$) postmenopausal women were cultured for 7 days in RPMI 1640 plus RANKL (25 ng/ml) and MCSF (100 ng/ml). Osteoclast-like cells were measured by TRAP positivity, expressed as mean $\pm$ SEM optical density (OD) units. Comparison between HIV- and HIV+ groups ($p=0.005$) met Bonferroni-adjusted criterion for significance ($p=0.0167$) as described in the "Statistical analysis" section

Fig. 2.

Induction of osteoclast-like cells by autologous sera: the autologous osteoclast induction assay. Peripheral blood adherent cells from HIV- ($N=51$) and HIV+ ($N=68$) postmenopausal women were cultured for 7 days in RPMI 1640 plus 15% autologous serum. Osteoclast-like cells were measured by TRAP positivity, expressed as mean $\pm$ SEM percent increase over background. Comparisons were made between HIV+ and HIV- subjects. Among HIV+ subjects, comparisons are made between subjects on or off antiretroviral therapy and between subjects on ritonavir-boosted protease inhibitor (PI)-based regimens (RTV+) and all regimens without RTV-boosted PIs (RTV-). Comparisons between HIV+ and HIV- groups ($p=0.01$) and RTV+ and RTV- groups ($p=0.0006$) met Bonferroni-adjusted criterion for significance ($p=0.0167$) as described in the “Statistical analysis” section

Fig. 3.

Association between autologous osteoclast induction (AOI) and N-telopeptide/TRAP5b and C-telopeptide/TRAP5b. Among HIV+ postmenopausal women, AOI is correlated with NTX/TRAP5b ($r=0.629$, $p<0.0001$) and CTx/TRAP5b ($r=0.452$, $p<0.01$)

Table 1

Characteristics of the study population (mean±SEM)

	HIV- (n=51)	HIV+ (n=68)	p value
Demographics and morphometry			
Age	59.3±1.0	56.0±0.7	<0.01
Race/ethnicity			NS
Hispanic (%)	65%	63%	
African American (%)	35%	37%	
BMI (kg/m ²)	30.5±0.8	27.8±0.8	<0.05
Body fat (%)	40.0±0.8	34.7±0.9	<0.0001
Truncal fat (%)	38.8±1.0	35.4±0.9	<0.05
Bone mineral density			
LS BMD (g/cm ²)	0.950±0.024	0.884±0.016	<0.05
LS T-score	-1.2±0.2	-1.8±0.1	<0.01
TH BMD (g/cm ²)	0.921±0.022	0.852±0.017	<0.05
TH T-score	-0.4±0.1	-1.0±0.1	<0.01
FN BMD (g/cm ²)	0.810±0.022	0.737±0.016	<0.01
FN T-score	-0.7±0.2	-1.3±0.1	<0.01
DR BMD (g/cm ²)	0.665±0.013	0.644±0.011	NS
DR T-score	-0.5±0.2	-0.8±0.2	NS
Medical history			
Years since menopause	11.6±1.2	8.1±0.7	<0.05
Current smoker	20%	24%	NS
Alcohol, >1 drink/day	2%	13%	<0.05
Intravenous drug use (ever)	10%	13%	NS
Hyperlipidemia	41%	21%	<0.05
Diabetes	24%	28%	NS
Hepatitis C virus seropositive	8%	22%	<0.05
Hepatitis B virus seropositive	0%	1%	NS
Statins (current)	31%	13%	<0.05
Past glucocorticoids (ever)	8%	21%	NS

	HIV- (n=51)	HIV+ (n=68)	p value
HIV-related			
AIDS diagnosis		52%	
CD4+ T cell count (cells/mm ³)		533±42	
Nadir CD4+ T cell count		211±26	
HIV-1 viral load <400 copies/ml ^a		78%	
HIV-1 viral load <50 copies/ml ^a		43%	
PI-based regimens		46%	
NNRTI-based regimens		28%	
NRTI-only regimens		7%	
PI+NNRTI-based regimens		3	

^aValues for those on antiretroviral therapy

Table 2
Calcitropic and gonadal hormones, cytokines, and bone turnover markers (mean±SEM)

	HIV- (N=51)	HIV+ (N=68)	HIV+ ART- (N=11)	HIV+ ART+ (N=57)	ART+ RTV- (N=37)	ART+ RTV+ (N=20)
Estrone (pg/ml)	30.2±1.9	25.7±1.4	30.2±4.1	24.8±1.4	23.7±1.9	26.9±2.0
PTH (pg/ml)	42.5±2.7	37.2±2.3	44.1±5.7	35.8±2.4	36.2±3.2	35.2±3.8
25-OHD (ng/ml)	20.4±1.5	25.1±1.6*	18.9±3.6	26.3±1.8	26.8±2.3	25.4±2.9
1,25(OH) ₂ D (pg/ml)	41.9±2.4	35.6±1.8*	35.3±5.2	35.7±1.9	33.4±2.3	39.9±3.5
Osteocalcin (ng/ml)	5.8±0.3	7.3±0.4**	5.1±0.9	7.7±0.4****	7.0±0.4	9.0±0.8*****
BAP (U/L)	33.8±1.9	41.4±2.6	33.7±8.8	42.9±2.5	42.5±2.8	43.7±5.2
NTx (nmol/BCE/L)	17.6±0.9	24.0±1.4***	19.6±1.8	24.8±1.6*****	23.0±1.7	28.3±3.2
CTx (ng/ml) ^a	0.42±0.03	0.71±0.06***	0.44±0.07	0.76 ±0.07*****	0.71±0.09	0.84±0.10
TRAP5b (U/L) ^a	2.9±0.2	3.6±0.2*	2.7±0.2	3.7±0.2	3.9±0.3	3.3±0.4
IL-6 (pg/ml)	2.6±0.4	2.7±0.4	4.5±1.4	2.3±0.3	2.1±0.2	2.7±0.8
TNF-α (pg/ml) ^a	22.7±2.0	40.2±3.8***	40.9±5.3	40.0±4.4	40.0±5.0	40.2±8.6
RANKL (pg/ml) ^a	48.9±3.9	55.8±2.6	62.0±7.8	54.5±2.7	56.8±3.1	49.7±5.1
OPG (pg/ml) ^a	777±27	4,122±306***	2,946±193	4,274±335*****	4,106±354	4,850±876

Normal ranges: PTH, 14–66 pg/ml; 25OHD, 32–100 ng/ml; 1,25(OH)₂D, 15–67 pg/ml; osteocalcin 2.4–10.0 ng/ml; BAP, 11.6–29.6 U/L; NTx, 6.2–19.0 nM BCE/L; CTx, 0.112–0.738 ng/ml; TRAP 5b, 1.49–4.89 U/L; IL-6, 0.4–10.0 pg/ml

Comparisons presented in *italics* are statistically significant after adjustment for multiple comparisons as detailed in “Statistical analysis” section *HIV+ ART- HIV+ not receiving antiretroviral therapy, HIV + ART+ HIV+ receiving antiretroviral therapy, RTV+ rionavir (RTV)-boosted protease inhibitor (PI), RTV- any antiretroviral regimen not including RTV-PIs*

* $p<0.05$ (comparison between HIV- and HIV+);

** $p<0.01$ (comparison between HIV- and HIV+);

*** $p<0.001$ (comparison between HIV- and HIV+);

**** $p<0.05$ (comparison between HIV+ ART- and HIV+ ART+);

***** $p<0.01$ (comparison between HIV+ ART- and HIV+ ART+);

***** $p<0.05$ (comparison between ART+ RTV- and ART+ RTV+)

^a Certain measures were not available for all subjects: TNF-α and RANKL, $n=62$ HIV+ and 41 HIV; CTx, $n=53$ HIV+ and 47 HIV-; TRAP5b, $n=40$ HIV+ and 32 HIV-; OPG, $n=35$ HIV+ and 35 HIV-