

# Antiretroviral therapy induces a rapid increase in bone resorption that is positively associated with the magnitude of immune reconstitution in HIV infection

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**Objective:** Antiretroviral therapy (ART) paradoxically intensifies bone loss in the setting of HIV infection. Although the extent of bone loss varies, it occurs with virtually all ART types, suggesting a common pathway that may be aligned with HIV disease reversal. Using an animal model of immunodeficiency we recently demonstrated that immune activation associated with CD4<sup>+</sup> T-cell reconstitution induces increased production of the osteoclastogenic cytokines RANKL and TNF $\alpha$  by immune cells, driving enhanced bone resorption and loss in bone mineral density.

**Design:** To confirm these findings in humans, we investigated the early kinetics of CD4<sup>+</sup> T-cell recovery in relation to biomarkers of bone turnover and osteoclastogenic regulators in a prospective 24-week cohort study.

**Methods:** Clinical data and blood sampling for HIV-RNA PCR, CD4<sup>+</sup> T-cell counts, bone turnover biomarkers, and osteoclastogenic regulators were obtained from ART-naïve HIV-infected study participants initiating standard doses of lopinavir/ritonavir plus tenofovir disoproxil fumarate/emtricitabine at baseline and at weeks 2, 8, 12, and 24 post ART.

**Results:** C-terminal telopeptide of collagen (CTX) a sensitive biomarker of bone resorption rose by 200% above baseline at week 12, remaining elevated through week 24 ( $\alpha < 0.01$ ), and was associated with significant increases in plasma levels of osteoclastogenic regulators [receptor activator of NF- $\kappa$ B ligand (RANKL), tumor necrosis factor alpha, (TNF $\alpha$ )]. Importantly, the magnitude of CD4<sup>+</sup> T-cell recovery correlated significantly with CTX ( $r_s = 0.387$ ,  $\alpha = 0.01$ ).

**Conclusion:** Our data suggest that ART-induced bone loss occurs early, is aligned with early events of immune reconstitution, and these immune changes provide a unifying mechanism to explain in part the skeletal decline common to all ART.

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## Introduction

It has long been recognized that HIV infection and antiretroviral therapy (ART) lead to significant deterioration of the adult skeleton [1–7] and may prevent attainment of optimal peak bone mineral density (BMD) in young men infected with HIV early in life [8]. Recent studies report osteopenia in up to two thirds, and outright osteoporosis in up to 15% of HIV-infected patients [9–11]. With the aging of the HIV/AIDS population, higher prevalence of low BMD has been reported in postmenopausal women infected with HIV that could place them at high risk for future fractures [12]. Furthermore, recent large observational studies reveal that compared with the general population there is a 2–4 fold higher prevalence of overall bone fracture in HIV-infected patients [13–16] and almost nine-fold increase in risk of hip fracture [17].

Bone fracture can have dire consequences [18]. Vertebral fractures are associated with deformity and/or chronic back pain. Clinical management of hip fracture almost always involves invasive surgical intervention, and long-term rehabilitation is needed in up to 75% of patients [19,20]. Mortality rates for hip fracture victims range between 24 and 32% in just the first year [21,22]. The economic cost of bone fractures is projected to reach \$25 billion per year by 2025 [23]. Despite the recent emergence of guidelines regarding screening, diagnosis, and treatment of bone disease in HIV populations [24,25], the utility of bone densitometry-based T scores, upon which clinical definitions of osteopenia and osteoporosis are based, remain unclear in HIV-infected populations. This uncertainty is further compounded in relatively younger study participants, as BMD derived T scores do not predict fracture incidence in study participants under 55 years of age [26]. Given these potential confounders, a better understanding of the underlying pathology by which HIV infection and ART promote bone loss is warranted.

The immuno-skeletal interface (ISI) is a centralization of shared cells and cytokine effectors that serve separate functions within both the immune and skeletal systems and includes monocytes/macrophages, B cells and T cells. Changes in the ISI may underlie the pathophysiology of diverse osteoporotic conditions including rheumatoid arthritis, periodontal infection, postmenopausal osteoporosis, hyperparathyroidism and even bone loss in HIV infection [27]. In fact, we recently demonstrated in both a murine model and human studies that HIV-induced altered B-cell function resulted in a significant increase in B-cell production of RANKL, the key osteoclastogenic factor, and a simultaneous decline in its physiological antagonist, OPG. This imbalance in the RANKL/OPG ratio may drive bone resorption and loss of BMD associated with HIV infection [28,29].

Perhaps more puzzling is the finding that ART – meant to suppress viral replication, increase the CD4<sup>+</sup> T-cell count, and reverse the consequences of HIV infection – actually worsens bone loss resulting in additional BMD loss of 2–6% within the first 2 years of treatment [1–3,24,30,31]. This phenomenon appears to be common to all ART types although varying in degree for different regimens, suggesting an indirect but common pathway for ART-induced bone loss [32–34] rather than just a direct effect of ART drugs on bone cells.

Chronic inflammation is a key feature of HIV infection even when well controlled with ART and has been implicated in the increasing prevalence of end-organ diseases in the aging HIV/AIDS population [35]. Interestingly, chronic inflammation associated with CD4<sup>+</sup> T-cell activation is an established mechanism for bone loss in multiple pathological situations including periodontal disease and postmenopausal osteoporosis [36–40]. Because CD4<sup>+</sup> T-cell repopulation can induce inflammation and/or immune activation, we speculated that HIV disease reversal and the associated repopulation of CD4<sup>+</sup> T cells following ART initiation might provide a unifying mechanism for ART-induced skeletal decline [6,41]. In fact, we recently demonstrated that T-cell reconstitution by adoptive transfer in the setting of prior immunodeficiency (TCR $\beta$  knockout mice), mimicking T-cell repopulation such as that seen with ART-initiation, initiates an inflammatory immune response leading to significant chronic loss of BMD and degradation of cortical and trabecular bone structure [42].

To explore this phenomenon further, in the current study, we assessed the relationship between biomarkers of bone resorption, osteoclastogenic cytokines, and CD4<sup>+</sup> T-cell recovery following ART initiation in treatment naïve HIV-infected adult patients.

## Materials and methods

### Study population

The current study was conducted with repurposed samples from an unrelated study. The original study was a single center, prospective cohort study in which study participants were recruited from the Grady Infectious Diseases Programme outpatient clinic in Atlanta, Georgia. Eligibility criteria included viremic ART-naïve men and women HIV-infected study participants, age at least 18 years. Potential study participants were excluded if they were on investigational drugs, had active opportunistic infection, had renal/hepatic impairment, or were pregnant.

### Study procedures

At enrollment, demographic information and clinical laboratory data including HIV-1 RNA PCR, CD4<sup>+</sup>

T-cell counts were obtained. Baseline samples were collected for biomarkers of bone turnover [C-terminal telopeptide of collagen (CTX) and osteocalcin] and osteoclastogenic regulators (RANKL, OPG, and TNF $\alpha$ ). Study participants initiated therapy with a fixed dose formulation of lopinavir/ritonavir 400/100 mg BID and a fixed dose formulation of tenofovir disoproxil fumarate/emtricitabine 300/200 mg once daily, a regimen that was commonly used in our practice at the time of this study. Blood samples for study endpoints were again obtained at weeks 2, 8, 12, and 24. This study was designed according to the ethical guidelines for human studies and approved by the Institutional Review Board of Emory University. All study participants enrolled in the parent study consented to the use of their samples in other HIV-related studies.

### Biochemical indices of bone turnover

Blood samples used in this study were processed within 60 min of collection and plasma was separated by centrifugation and frozen at  $-80^{\circ}\text{C}$  until analysis. On the day before analysis, samples were thawed at  $4^{\circ}\text{C}$  overnight. Commercial enzyme-linked immunosorbent assays (ELISA) were used to quantify plasma CTx and osteocalcin (Immunodiagnostic Systems, Scottsdale, Arizona, USA) [43,44], and total soluble RANKL, OPG (Alpco Diagnostic, Salem, New Hampshire, USA), and TNF $\alpha$  (R&D Systems, Minneapolis, Minnesota, USA) [45,46]. The lower limits of quantification for these assays were 0.02 ng/ml for CTx, 0.11 ng/ml for osteocalcin, 1.56 pg/ml for RANKL, 1.4 pg/ml for OPG, and 1.6 pg/ml for TNF $\alpha$ .

### Statistical analysis

Statistical significance was determined using GraphPad Prism 5.0 for Mac (GraphPad Software Inc. La Jolla, California, USA). Data was analyzed using nonparametric tests unless they passed the D'Agostino & Pearson omnibus normality test. Longitudinal data was analyzed by repeated measures analysis of variance (ANOVA) using Tukey's multiple comparison *post hoc* test for parametric data. For nonparametric data we used the Friedman repeated measures ANOVA with Dunn's multiple comparison *post hoc* test. All correlations involved Spearman test for nonparametric data.  $P < 0.05$  was considered statistically significant. In some studies observations were missing for one or more patients for one or more time points and the study participants were excluded in order to perform repeated measures ANOVA.

To enable prospective statistical analysis by repeated measures ANOVA nondetectable values were imputed for some endpoints using the L/2 substitution formulae where L is the limit of detection as described [47]. For viral loads the limit of detection (LD) is 400 copies/ $\mu\text{l}$  and  $L/2 = 200$ . For TNF $\alpha$  limit of detection = 0.15 pg/ml and  $L/2 = 0.075$ .

## Results

### Study participant demographics and characteristics

Of the 20 enrolled study participants, 90% were African American, 80% were men, and the mean age was 39.5 years. The mean entry HIV-1 RNA PCR and CD4 $^{+}$  T-cell counts were 138 500 copies/ml and 148 cells/ $\mu\text{l}$  respectively, reflecting the HIV disease severity of the cohort. Detailed demographic characteristics, baseline clinical and laboratory profile, and antiretroviral regimens are summarized in Table 1.

### Virologic suppression is associated with a rapid increase in CD4 $^{+}$ T cells

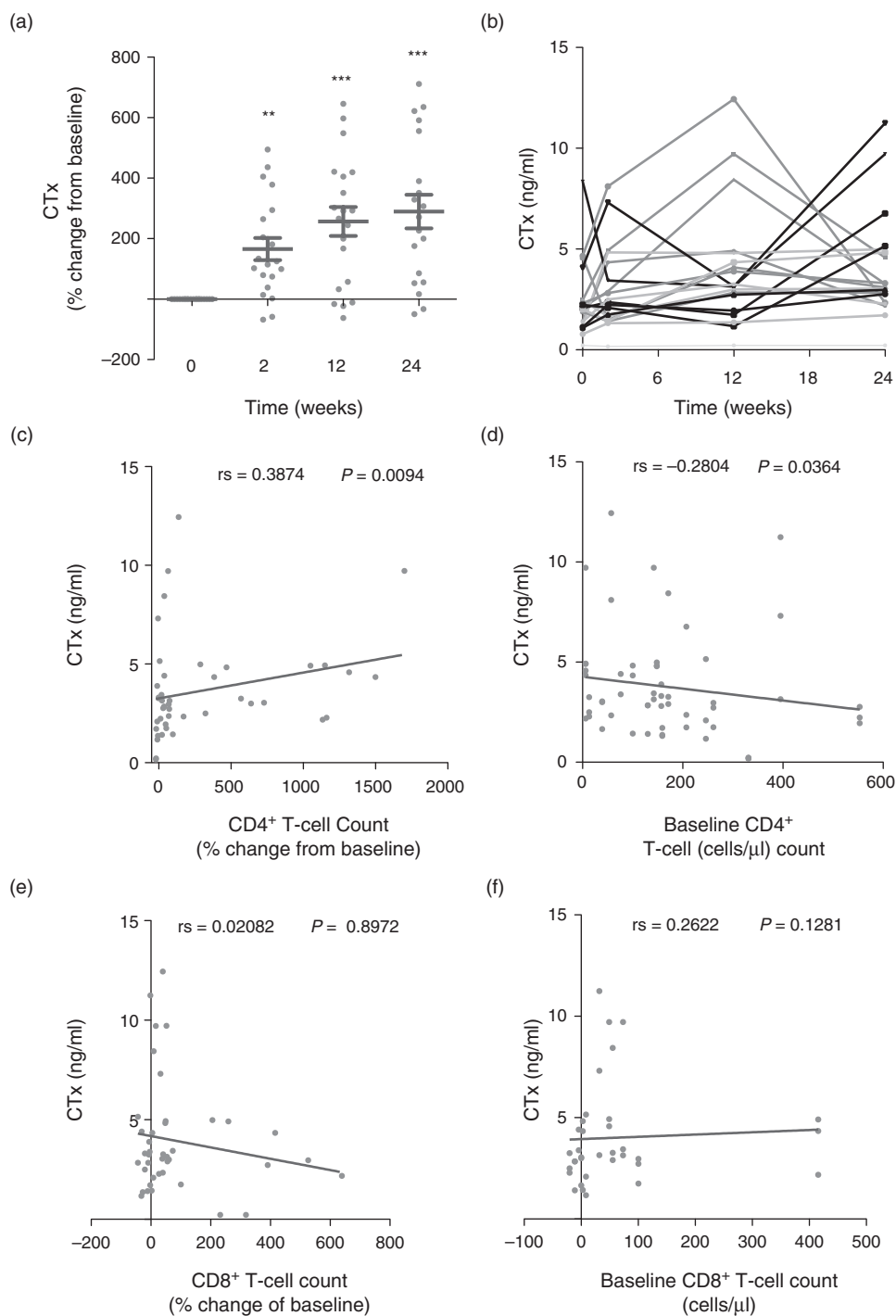
As expected, ART initiation led to a rapid suppression of HIV with 19 of the 20 study participants achieving undetectable plasma viral load ( $<400$  copies/ml) at 24 weeks (Supplemental Figures 1A and B, <http://links.lww.com/QAD/A797>). Correspondingly, CD4 $^{+}$  T cell-counts increased from a baseline mean of 148 cells/ $\mu\text{l}$  by an average of 71% within 2 weeks of ART and  $\sim 80\%$  by 24 weeks (Supplemental Figures 1C and D, <http://links.lww.com/QAD/A797>).

ART initiation causes an overall rapid rise in bone resorption that is positively correlated with the magnitude of CD4 $^{+}$  T-cell increase and negatively correlated with baseline CD4 $^{+}$  T-cell number. Within just 2 weeks of ART initiation, mean plasma CTx increased significantly from baseline levels and remained elevated for at least 24 weeks (Fig. 1a). Importantly, individual patient responses were extremely heterogeneous with about 25% of patients achieving a robust peak increase in CTx of up to 600% of baseline.

In order to track the kinetics of CTx change, CTx (ng/ml) was plotted over time for each patient. The data

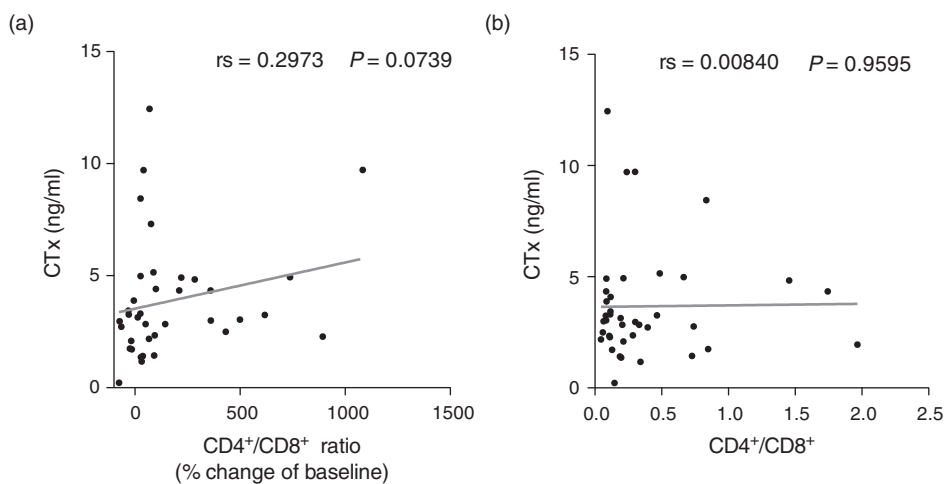
**Table 1. Baseline demographic and clinical characteristics.**

Characteristics	Group (n = 20)
Gender	
Male n (%)	16 (80)
Female n (%)	4 (20)
Race	
African American n (%)	18 (90)
White (non-Hispanic) n (%)	2 (10)
Antiretroviral regimen:	
Lopinavir/ritonavir + tenofovir + emtricitabine n (%)	20 (100)
Other demographics mean (IQR)	
Age (years)	39.5 (34.0, 44.0)
Weight (kg)	70.7 (63.7, 78.2)
Body mass index	23.7 (21.7, 25.4)
HIV-RNA PCR (copies/ml)	138 500 (32 200, 357 250)
CD4 $^{+}$ T-cell counts (cells/ $\mu\text{l}$ )	148.0 (57.0, 246.0)
CD8 $^{+}$ T-cell counts (cells/ $\mu\text{l}$ )	841.0 (262.0, 1101.0)
Creatinine clearance (ml/min)	110.6 (80.5, 133.9)
Alanine amino transferase (mg/ml)	39.0 (22.5, 37.0)
Aspartate amino transferase (mg/ml)	48.0 (22.0, 54.0)



**Fig. 1. ART elicits a rapid increase in bone resorption that correlates positively with the magnitude of CD4<sup>+</sup> T-cell repopulation.**

(a) Plasma CTx (% change from baseline) and (b) CTx (ng/ml) was quantified at baseline (0) and 2, 12 and 24 weeks after ART initiation. \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  vs. baseline by Friedman repeated measures ANOVA and Dunn's multiple comparison *post hoc* test. Red dots represent individual patients and blue bars represent mean  $\pm$  SE. For each patient at 2, 12 and 24 weeks of ART, the rate of bone resorption (CTx) was correlated to (c) the magnitude of CD4<sup>+</sup> T-cell change, (d) the baseline CD4<sup>+</sup> T-cell count (e) the magnitude of CD8<sup>+</sup> T-cell change, and (f) the baseline CD8<sup>+</sup> T-cell count. Spearman rank correlation coefficient ( $r_s$ ) and significance level ( $P$ ) are shown.



**Fig. 2. Bone resorption is positively associated with the magnitude of change in CD4<sup>+</sup>/CD8<sup>+</sup> ratio after ART initiation.** For each patient at 2, 12 and 24 weeks of ART the rate of bone resorption (CTx) was correlated to (a) the magnitude of CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio change and (b) the absolute CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio. Spearman rank correlation coefficient ( $r_s$ ) and significance level ( $P$ ) are shown. Red dots represent individual patients.

reveal (Fig. 1b) that in some patients, resorption rose immediately and peaked by 12 weeks, declining thereafter (red lines), whereas others were delayed, rising after 12 weeks of ART and persisting for at least 24 weeks (black lines). One patient had undetectable CTx values at all time points (yellow line).

We hypothesize that an inflammatory state initiated by CD4<sup>+</sup> T-cell repopulation/immune-reaktivation following initiation of ART causes a direct increase in bone resorption. In support of this hypothesis we found a significant ( $P=0.0094$ ) correlation ( $r_s=0.3874$ ) between the magnitude of CD4<sup>+</sup> T-cell reconstitution (percentage change from baseline) and the extent of bone resorption (CTx) after ART initiation (Fig. 1c). Furthermore, there was a significant ( $P=0.0364$ ) negative association ( $r_s=-0.2804$ ) between bone resorption (CTx) and the baseline CD4<sup>+</sup> T-cell count (Fig. 1d) consistent with a recent report of low baseline CD4<sup>+</sup> T-cell count associated with greater loss of BMD after ART initiation [48]. In contrast, the magnitude of the change in CD8<sup>+</sup> T cells (percentage change from baseline) did not correlate significantly with changes in CTx following ART (Fig. 1e). There was also no significant association between the baseline CD8<sup>+</sup> T-cell count and CTx concentrations following ART (Fig. 1f).

Another measure of the robustness of immune reconstitution is the change in CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio. To investigate an association between the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio and bone resorption we correlated CTx with the magnitude of the change in CD4<sup>+</sup>/CD8<sup>+</sup> ratio (percentage change from baseline). Interestingly the data revealed (Fig. 2a) a strong positive trend between bone resorption and CD4<sup>+</sup>/CD8<sup>+</sup> ratio ( $r_s=0.2973$ ), which fell just short of statistical significance ( $P=0.0739$ ). Not

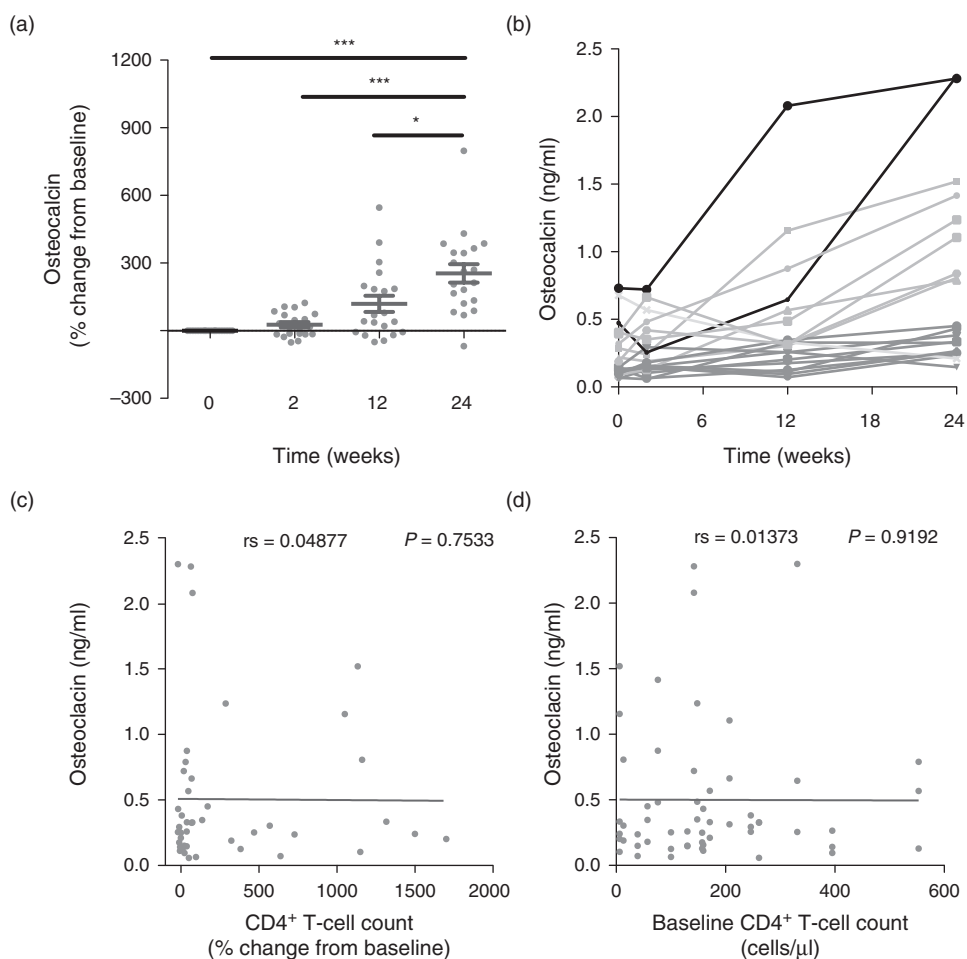
surprisingly, without baseline normalization there was no association between absolute CD4<sup>+</sup>/CD8<sup>+</sup> ratio and bone resorption (Fig. 2b).

### Increased bone resorption after antiretroviral therapy initiation leads to a compensatory increase in bone formation

Osteocalcin is secreted at high concentrations by mineralizing osteoblasts and is a sensitive and specific biochemical marker of *in vivo* bone formation. In concert with rising rates of bone resorption, aggregate plasma osteocalcin was observed to rise significantly in most patients after ART initiation likely as a compensatory response to increased resorption (Fig. 3a). As with CTx, individual patient responses were heterogeneous (Fig. 3b) with two patients undergoing large sustained increases in osteocalcin (black lines). Seven more study participants underwent robust responses (purple lines) whereas the remainder (red lines) underwent only small changes in bone formation rate. One patient underwent a persistent decline in osteocalcin from baseline after ART initiation over the 24 weeks (green line). Unlike CTx that correlated with the magnitude of CD4<sup>+</sup> T-cell repopulation after ART, osteocalcin showed no association with CD4<sup>+</sup> T-cell recovery (Fig. 3c). There was also no association between post-ART osteocalcin levels and baseline CD4<sup>+</sup> T-cell count (Fig. 3d).

### Antiretroviral therapy initiation is accompanied by increased production of osteoclastogenic cytokines

In order to account for the elevated indices of bone resorption, we quantified total plasma levels of the key osteoclastogenic cytokine, RANKL. To normalize for very high variability in baseline RANKL concentrations between patients, we calculated percentage change from



**Fig. 3. ART elicits a compensatory increase in bone formation.** (a) Plasma osteocalcin (% change from baseline) and (b) osteocalcin (ng/ml) was quantified at baseline (0) and 2, 12 and 24 weeks after ART initiation. \* $P < 0.05$  or \*\*\* $P < 0.001$  by Friedman repeated measures ANOVA and Dunn's multiple comparison *post hoc* test. For each patient at 2, 12 and 24 weeks of ART the rate of bone formation (osteocalcin) was correlated to (c) the magnitude of CD4<sup>+</sup> T-cell change and (d) the baseline CD4<sup>+</sup> T-cell count. Spearman rank correlation coefficient ( $r_s$ ) and significance level ( $P$ ) are shown. Red dots represent individual patients and blue bars represent mean  $\pm$  SE.

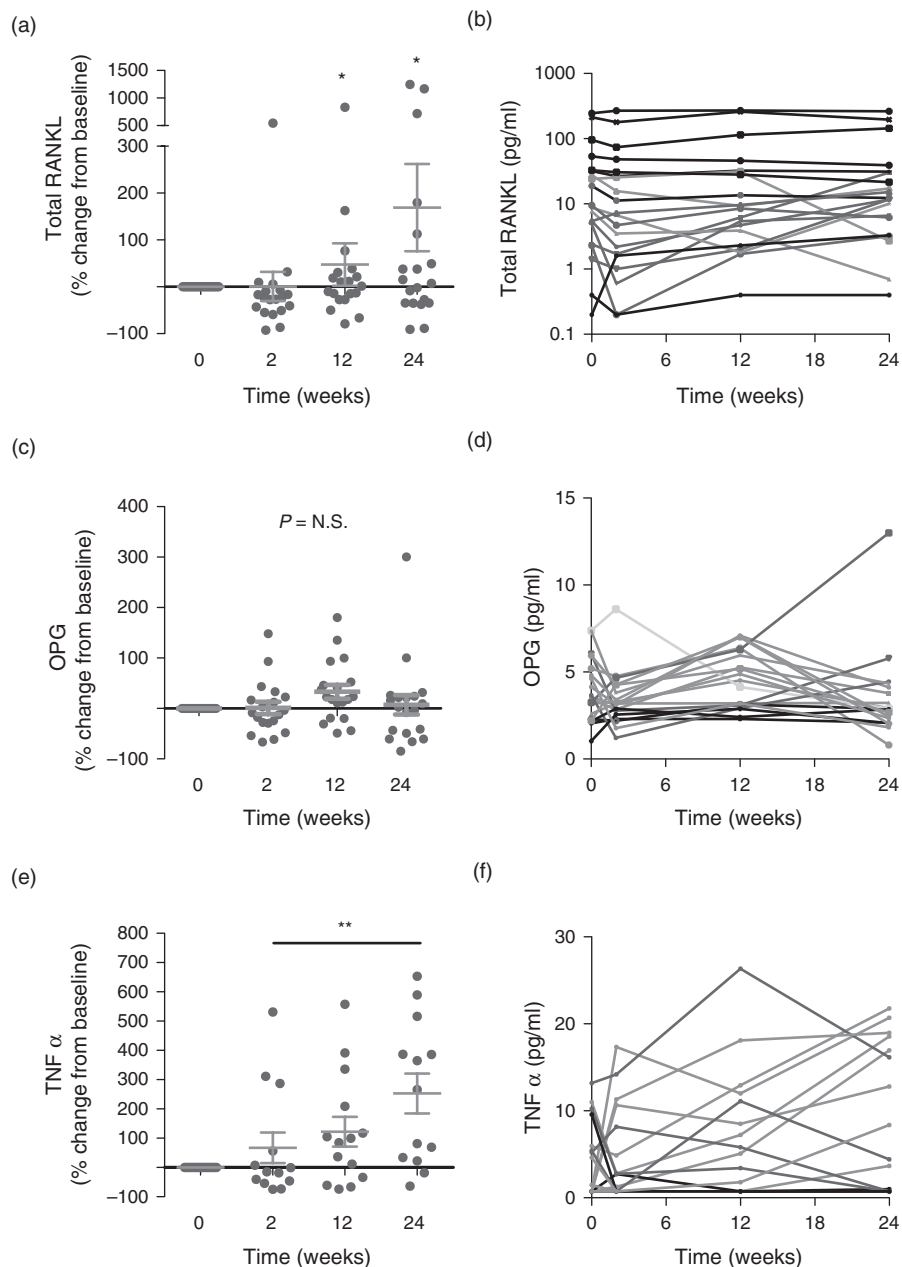
baseline for each patient for 2, 12 and 24 weeks of ART (Fig. 4a), revealing significant increases in RANKL production by 12 and 24 weeks of ART.

When absolute RANKL concentrations (pg/ml) were plotted for individual patients, study participants with the highest baseline RANKL production (black lines) did not vary much following ART initiation (Fig. 4b), possibly because their RANKL production may have been near maximal due to HIV-associated bone resorption. In contrast, 30% of patients, almost exclusively the lowest RANKL producers at baseline, had dramatic increases in RANKL by 12 and 24 weeks (red lines). Because some patients exhibited extraordinarily high levels of RANKL that did not change over the 6 months of the study, the mean RANKL change for all patients underrepresented the important changes occurring in some subsets of patients. These data highlight the importance of

evaluating human study participants on an individual basis, rather than simply in aggregate.

Plasma levels of OPG, the RANKL decoy receptor, were not significantly altered overall at any time point (Fig. 4c) although fluctuations were evident for individual patients (Fig. 4d), with three patients undergoing some increase (red lines), but with most study participants undergoing declines in OPG at 24 weeks (blue lines). Overall increased RANKL production with no significant compensatory increase in OPG would lead to conditions suitable for osteoclastic bone loss as observed in many patients (Fig. 1a and b).

In our previous studies in rodents we identified significant upregulation in TNF $\alpha$ , an inflammatory cytokine that can induce RANKL production [49] and amplify its osteoclastogenic activity [50–52]. Under normal basal



**Fig. 4. The osteoclastogenic factors RANKL and TNF $\alpha$  are significantly increased following ART initiation.** (a) Percentage change in plasma RANKL, (b) absolute change in plasma RANKL, (c) percentage change in plasma OPG, (d) absolute change in plasma OPG, (e) percentage change in plasma in TNF, and (f) absolute change in plasma TNF were quantified at baseline (0 time) and 2, 12 and 24 weeks of ART. \* $P < 0.05$  or \*\* $P < 0.01$  vs. baseline by Friedman repeated measures ANOVA and Dunn's multiple comparison *post hoc* test.  $P = NS$  (not significant). Red dots represent individual patients and blue bars represent mean  $\pm$  standard error.

conditions, plasma TNF $\alpha$  levels are undetectable with our commercial ELISA assay system; however, TNF $\alpha$  was detectable (and thus elevated) in eight of 20 study participants at baseline, likely as a consequence of inflammation associated with HIV infection. Following ART initiation, plasma TNF $\alpha$  levels rose in some, but not all patients, beginning as early as 2 weeks, with a significant 150% overall increase from baseline at 24 weeks (Fig. 4e). Prospective changes are shown as actual

concentrations (pg/ml) for patients with detectable levels at one or more time points in Fig. 4f.

## Discussion

The mechanism by which ART exacerbates HIV-induced skeletal decline remains unclear. Chronic inflammation in HIV patients, even in the context of effective viral

suppression by ART, has been well documented and suggested to contribute to the metabolic complications of treated chronic HIV infection [35]. In fact, inflammation has long been recognized as a driver of bone turnover and loss in multiple diverse osteoporotic conditions including alveolar bone loss in periodontal infection, focal and systemic bone erosions in the inflammatory autoimmune disease rheumatoid arthritis, and in estrogen deficiency associated bone loss that underlies postmenopausal osteoporosis [40]. As a consequence we have proposed that inflammation associated with T-cell and/or immune reconstitution may contribute to bone loss following ART initiation [6,41]. Recently, we tested this concept by reconstituting T cells by adoptive transfer into T-cell-deficient TCR $\beta$  knockout mice and demonstrated that T-cell repopulation and reactivation of the adaptive immune response did indeed lead to a significant loss of BMD and trabecular and cortical bone mass. Bone loss was a consequence of a significant upregulation in osteoclastic bone resorption resulting from elevated production of RANKL and TNF $\alpha$  by T cells, with additional contributions from other immune cells, primarily B cells and macrophages [42].

In the present study, we have translated these concepts into the human system by examining the early effects of ART initiation on bone turnover and osteoclastogenic cytokine production in HIV-infected ART naïve patients. Our data reveal a rapid increase in bone resorption (measured by CTx) in HIV patients initiating ART, beginning as early as 2 weeks and plateauing between 12 and 24 weeks post ART initiation. This rise occurred concurrently with virologic suppression and CD4<sup>+</sup> T-cell repopulation, and was accompanied by increased serum levels of plasma TNF $\alpha$  and RANKL. These rapid changes in TNF $\alpha$  and RANKL without significant compensation by OPG are remarkably consistent with changes in osteoclastic modulators observed in our animal study of immune immunoreconstitution bone loss [42]. Taken together, the significant correlation between the magnitude of CD4<sup>+</sup> T-cell repopulation following ART and the rate of bone resorption elicited, support an association between T-cell recovery, immune activation, and bone resorption with ART.

In HIV-infected patients, suppression of HIV replication with ART leads to a partial recovery of depleted CD4<sup>+</sup> T cells through mechanisms involving both peripheral expansion of existing T-cell pools and interleukin-7-mediated thymic reactivation pathways [53,54]. Furthermore, CD4<sup>+</sup> T cells are central regulators of adaptive immunity and potentially affect the action of other immune cells, including professional antigen presenting cells such as dendritic cells, macrophages and B cells, all of which are capable of secreting TNF $\alpha$  and/or RANKL. Presumably therefore, activation of the expanding CD4<sup>+</sup> T-cell pool, together with the reactivation of

antigen presenting cells during ART-induced disease reversal leads to excessive production of key osteoclastogenic cytokines including TNF $\alpha$  and RANKL, tilting the RANKL/OPG balance in favor of enhanced bone breakdown.

Interestingly, increased plasma osteocalcin signaled a significant increase in bone formation likely as a compensatory response to elevated bone resorption. This coupling response may significantly ameliorate the extent of bone loss in some patients, although our studies revealed that high rates of bone resorption were not matched by equivalent robust increases in bone formation in many patients and such patients may be at particularly high risk for bone loss and development of osteoporosis. In other cases robust changes in bone formation accompanied relatively small or modest increases in bone resorption and these study participants may be better protected from bone loss. It should however be pointed out that the stoichiometry of changes in metabolic turnover markers is currently not clear and it is unknown what magnitude of change in osteocalcin is needed to offset a particular change in CTx, or even if the association is linear.

It has been reported that patients with low baseline CD4<sup>+</sup> cell count have greater bone loss following ART initiation [48]. Our studies verify this response and show a significant negative correlation between basal CD4<sup>+</sup> T-cell count and CTx concentrations after ART. Mechanistically, although unclear why this is the case, we hypothesize that these observations may suggest that the extent of homeostatic repopulation may have an impact on bone turnover. Interestingly, in our mouse model we did indeed find that relatively fewer transplanted CD4<sup>+</sup> T cells elicited greater bone loss than very large numbers [42].

The limitations of our study include the small sample size and the brevity of the 24-week clinical follow-up period that limited our ability to capture the effect of ART-induced disease reversal and immune reconstitution on the skeleton over a longer period of time. Nonetheless, the magnitude and the robustness of bone resorption following ART initiation allowed significant changes to be observed with the cohort size examined.

As several previous studies had examined the effects of ART on bone at the later stage (>6 months) of therapy, our focus was directed at evaluating how early these effects begin and their relationship with the early events of immune reconstitution and bone turnover. It is well established that BMD decline follows ART initiation and our studies reveal that ART-initiates bone turnover providing a mechanistic basis for these later changes.

It is worth noting that participants in our clinical study were overwhelmingly African American men with



advanced HIV/AIDS. This population is completely representative of the demographic treated at our Atlanta site and southern United States where the HIV/AIDS epidemic is still a major problem among minority population. Although other studies that include participants from both sexes with diverse racial and ethnic background have all demonstrated bone loss, additional studies will be needed to assess the generalizability of our findings to other racial groups and in women.

In conclusion, our data demonstrate that bone loss begins almost immediately following ART initiation in humans and is associated with repopulation of T cells. ART-associated bone loss may thus be related, in large part, to immune regeneration/reactivation rather than, or in addition to, direct effects of ART on bone cells. As the present study cannot exclude a direct effect of ART, additional mechanistic studies are warranted to demonstrate a cause effect relationship between bone loss, CD4<sup>+</sup> repopulation and immune recovery. Furthermore, short-term antiresorptive therapy to prevent bone loss associated with T-cell repopulation during the early reconstitution period should be explored further in HIV-infected patients starting ART.

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## Roles of authors

I.O. and M.N.W. were involved in the study conception, design, and implementation, and writing of the manuscript. K.T., A.V., S.R.P., T.V. performed all the experiments and were involved in data analysis. F.V., K.R., A.N.S., C.D.L., J.L.L. were involved in study conception, design, and significant editing of the manuscript.

## Conflicts of interest

There are no conflicts of interest.

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