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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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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Abstract

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+ T cell reconstitution induces increased production of the osteoclastogenic cytokines RANKL and TNFα 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+ 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, CD 4+ T cell counts, bone turnover biomarkers, and osteoclastogenic regulators were obtained from ART-naïve HIV-infected subjects 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 (RANKL and TNF\(\alpha\)). Importantly, the magnitude of CD4+ T cell recovery correlated significantly with CTx (\(Rs=0.387, \alpha=0.01\)).

**Conclusions**—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 the skeletal decline common to all ART.

**Keywords**
Osteoporosis; antiretroviral therapy; ART; HAART; cART; osteoclasts; bone loss; T cells; B cells; HIV; RANKL; TNF\(\alpha\)

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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 to the general population there is a 2–4 fold higher prevalence of overall bone fracture in HIV-infected patients [13–16] and almost 9 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 subjects, as BMD derived T scores do not predict fracture incidence in subjects 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+ 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+ T cell activation is an established mechanism for bone loss in multiple pathological situations including periodontal disease and postmenopausal osteoporosis [36–40]. Because CD4+ T cell repopulation can induce inflammation and/or immune activation, we speculated that HIV disease reversal and the associated repopulation of CD4+ 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 (TCRβ knock out 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+ T cell recovery following ART initiation in treatment naive 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 subjects were recruited from the Grady Infectious Diseases program outpatient clinic in Atlanta, Georgia. Eligibility criteria included viremic ART-naïve male and female HIV-infected subjects age ≥18 years. Potential subjects 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+ T cell counts were obtained. Baseline samples were collected for biomarkers of bone turnover (CTx and osteocalcin) and osteoclastogenic regulators (RANKL, OPG, and TNFα). Subjects initiated therapy with a fixed dose formulation of lopinavir/ritonavir 400/100 mg BID plus a fixed dose formulation of tenofovir disoproxil fumarate/emtricitabine 300/200 mg QD, 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 subjects 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 minutes of collection and plasma was separated by centrifugation and frozen at −80°C until analysis. On the day before analysis, samples were thawed at 4°C overnight. Commercial enzyme-linked immunosorbent assays (ELISA) were used to quantify plasma CTx and osteocalcin (Immunodiagnostic Systems, Scottsdale, AZ) [43, 44], and total soluble RANKL, OPG (Alpco Diagnostic, Salem, NH), and TNFα (R&D Systems) [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 TNFα.

Statistical Analysis

Statistical significance was determined using GraphPad Prizm 5.0 for Mac (GraphPad Software Inc. La Jolla, CA). Data was analyzed using nonparametric tests unless they passed the D’Agostino & Pearson omnibus normality test. Longitudinal data was analyzed by repeated measures ANOVA using Tukey’s Multiple Comparison post hoc test for parametric data. For non-parametric 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 subjects were excluded in order to perform repeated measures ANOVA.

To enable prospective statistical analysis by repeated measures ANOVA non-detectable values were imputed for some endpoints using the L/2 substitution formulae where L = the limit of detection (LD) as described [47]. For viral loads the LD is 400 copies/μl and LD/2 = 200. For TNFα LD = 0.15 pg/ml and L/2 = 0.075.

RESULTS

Study participant demographics and characteristics

Of the 20 enrolled subjects, 90% were African American, 80% were male, 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/μL 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 subjects achieving undetectable plasma viral load (<400 copies/ml) at 24 weeks (Supplemental Fig 1A and B). Correspondingly, CD4+ T cell counts increased from a baseline mean of 148 cells/μl by an average of 71% within 2 weeks of ART and ~80% by 24 weeks (Supplemental Fig 1C and D).

**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 reveal (Fig. 1B) that in some patients, resorption rose immediately and peaked by 12 weeks, declining thereafter (red lines), while 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+ T cell repopulation/immune-reactivation following initiation of ART initiation causes a direct increase in bone resorption. In support of this hypothesis we found a significant (P=0.0094) correlation (Rs=0.3874) between the magnitude of CD4+ 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 (Rs=−0.2804) between bone resorption (CTx) and the baseline CD4+ T cell count (Fig. 1D) consistent with a recent report of low baseline CD4+ T cell count associated with greater loss of BMD after ART initiation [48]. In contrast, the magnitude of the change in CD8+ 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+ T cell count and CTx concentrations following ART (Fig. 1F).

Another measure of the robustness of immune reconstitution is the change in CD4/CD8 T cell ratio. To investigate an association between the CD4/CD8 T cell ratio and bone resorption we correlated CTx with the magnitude of the change in CD4/CD8 ratio (percentage change from baseline). Interestingly the data revealed (Fig. 2B) a strong positive trend between bone resorption and CD4/CD8 ratio (Rs=0.2973), which fell just short of statistical significance (P=0.0739). Not surprisingly, without baseline normalization there was no association between absolute CD4/CD8 ratio and bone resorption (Fig. 2B).
Increased bone resorption after ART 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 \textit{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 subjects underwent robust responses (purple lines) while 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\(^+\) T cell repopulation after ART, osteocalcin showed no association with CD4\(^+\) T cell recovery (Fig. 3C). There was also no association between post-ART osteocalcin levels and baseline CD4\(^+\) T cell count (Fig. 3D).

\textbf{ART 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 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, subjects 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 subjects 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 3 patients undergoing some increase (red lines), but with most subjects 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 \cite{49} and amplify its osteoclastogenic activity \cite{50–52}. Under normal basal conditions, plasma TNF\(\alpha\) levels are undetectable with our commercial ELISA assay system; however TNF\(\alpha\) was detectable (and thus elevated) in 8 of 20 subjects at baseline, likely as a consequence of inflammation...
associated with HIV infection. Following ART initiation, plasma TNFα 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 Figure 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 system 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 system 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 TCRβ knock out 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 TNFα 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+ T cell repopulation, and was accompanied by increased serum levels of plasma TNFα and RANKL. These rapid changes in TNFα and RANKL without significant compensation by OPG are remarkably consistent with changes in osteoclastic modulators observed in our animal study of immune dysregulation-induced bone loss [42]. Taken together, the significant correlation between the magnitude of CD4+ 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+ T cells through mechanisms involving both peripheral expansion of existing T cell pools and IL-7–mediated thymic reactivation pathways [53, 54]. Furthermore, CD4+ T cells are central regulators of adaptive immunity and potently affect the action of other immune cells, including professional antigen presenting cells (APCs) such as dendritic cells, macrophages and B cells, all of which are capable of secreting TNFα and/or RANKL. Presumably therefore, activation of the expanding CD4+ T cell pool, together with the reactivation of APCs during ART-induced disease reversal leads to
excessive production of key osteoclastogenic cytokines including TNFα 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 subjects may be better protected from bone loss. It should however be pointed out that the stoichiometry of changes in metabolic turnover markers is presently 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 count have greater bone loss following ART initiation [48]. Our studies verify this response and show a significant negative correlation between basal CD4⁺ T cell count and CTx concentrations after ART. Mechanistically, while 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⁺ 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.

Since 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 males with advanced HIV/AIDS. This population is completely representative of the demographic treated at our Atlanta site and southern US 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 not
exclude a direct effect of ART, additional mechanistic studies are warranted to demonstrate a cause effect relationship between CD4 repopulation and immune recovery. Furthermore, short-term anti-resorptive 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

**ART** antiretroviral therapy
**BMD** bone mineral density
**CTx** C-terminal telopeptide of collagen
**DXA** dual energy X-ray absorptiometry
**FTC** emtricitabine
**LPV/r** lopinavir/ritonavir
**OPG** osteoprotegerin
**RANK** receptor activator of NF-κB
**RANKL** RANK ligand
**TCRβ** T cell receptorβ
**TDF** tenofovir disoproxil fumarate

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Figure 1. ART elicits a rapid increase in bone resorption that correlates positively with the magnitude of CD4\(^+\) 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 ± 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\(^+\) T cell change and (D) the baseline CD4\(^+\) T cell count. Spearman rank correlation coefficient (Rs) and significance level (P) are shown.
Figure 2. Bone resorption is positively associated with the magnitude of change in CD4/CD8 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/CD8 T cell ratio change and (B) the absolute CD4/CD8 T cell ratio. Spearman rank correlation coefficient (Rs) and significance level (P) are shown. Red dots represent individual patients.
Figure 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⁺ T cell change and (D) the baseline CD4⁺ T cell count. Spearman rank correlation coefficient (Rs) and significance level (P) are shown. Red dots represent individual patients and blue bars represent mean ± SE.
Figure 4. The osteoclastogenic factors RANKL and TNFα are significantly increased following ART initiation

The magnitude of change for each patient (percentage change from baseline) for (A) plasma RANKL, (C) plasma OPG and (E) plasma TNFα and absolute concentrations for each patient presented longitudinally for (B) plasma RANKL, (D) plasma OPG and (F) plasma TNFα, was 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 ± SE.
**Table 1**

Baseline demographic and clinical characteristics

<table>
<thead>
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<th>Characteristics</th>
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<tr>
<td>Gender</td>
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<tr>
<td>Male n (%)</td>
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<td>Female n (%)</td>
<td>4 (20)</td>
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<tr>
<td>Race</td>
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</tr>
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<td>African American n (%)</td>
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<tr>
<td>White (non-Hispanic) n (%)</td>
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<tr>
<td>Antiretroviral regimen: Lopinavir/ritonavir + tenofovir + emtricitabine n (%)</td>
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<td>Other demographics mean (IQR)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Weight (kg)</td>
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<td>HIV-RNA PCR (copies/ml)</td>
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<tr>
<td>CD4+ T-cell counts (cells/μl)</td>
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<tr>
<td>CD8+ T-cell counts (cells/μl)</td>
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<td>Creatinine clearance (ml/min)</td>
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<td>Aspartate amino transferase (mg/ml)</td>
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