Persistently lower bone mass and bone turnover among South African children living with well controlled HIV

Yanhan Shen[a], Stephanie Shiau[b], Renate Strehlau[c], Megan Burke[c], Faeezah Patel[c], Cara T. Johnson[d], Bridgette Rizkalla[e], Gallagher Dympna[e,f], Louise Kuhn[a,g], Ashraf Coovadia[c], Michael T. Yin[h] and Stephen M. Arpadi[a,g,i]

Objective: We evaluated longitudinal trends and associations between bone mass, bone turnover and inflammatory markers among South African children living with HIV (CLHIV) and controls.

Design: We previously reported decreased bone mass among CLHIV independent of marked inflammation and increased bone turnover. The goal of this study was to evaluate longitudinal changes in bone mass, bone turnover and inflammation over 2 years.

Methods: Longitudinal analyses were conducted among 220 CLHIV and 220 controls. Anthropometric measurements, physical activity, antiretroviral regimen, virologic and immunologic status, whole body (WB) and lumbar spine (LS) bone mineral content (BMC) and bone mineral density (BMD) were collected (enrollment, 12 and 24 months). Bone turnover markers including C-telopeptide of type I collagen (CTx) and procollagen type I N-terminal propeptide (P1NP) and inflammatory markers including interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), soluble CD14 and high-sensitivity C-reactive protein (hsCRP) were collected at enrollment and 24 months.

Results: Compared with controls, CLHIV had significantly lower mean WB-BMC, WB-BMD, WB-BMC *z* scores, LS-BMC and LS-BMD as well as lower bone formation (P1NP) and resorption (CTx), and higher hsCRP and soluble CD14 over 24 months. CLHIV on efavirenz (EFV) had consistently lower TNF-alpha and IL-6 compared with those on ritonavir-boosted lopinavir (LPV/r) at all time points.

Conclusion: Over 2 years of follow-up, South African CLHIV had persistently lower bone mass, bone turnover, and macrophage activation. Lower bone mass and higher pro-inflammatory cytokine profiles were consistently observed among those on LPV/r-based compared with EFV-based regimens.

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[a]Gertrude H. Sergievsky Center, Vagelos College of Physicians and Surgeons, Columbia University Irving Medical Center, New York, NY, [b]Department of Biostatistics and Epidemiology, Rutgers School of Public Health, Rutgers University, Piscataway, NJ, USA, [c]Empilweni Services and Research Unit, Rahima Moosa Mother and Child Hospital, Department of Paediatrics and Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, [d]Office of Scholarship & Research, School of Nursing, [e]New York Nutrition Obesity Research Center, Division of Endocrinology, Department of Medicine, [f]Institute of Human Nutrition, Vagelos College of Physicians and Surgeons, [g]Department of Epidemiology, Mailman School of Public Health, [h]Division of Infectious Disease, Department of Medicine, Vagelos College of Physicians & Surgeons, and [i]Department of Pediatrics, Vagelos College of Physicians & Surgeons, Columbia University Irving Medical Center, New York, NY, USA.

Correspondence to Stephen M. Arpadi, MD, MS, Gertrude H. Sergievsky Center, Columbia University, 630W. 168th Street, PH 19-114, New York, NY 10032, USA.

Tel: +1 212 305 2384; fax: +1 212 305 2526; e-mail: sma2@columbia.edu
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Introduction

Survival into adulthood is increasingly common for individuals with perinatally acquired HIV who are maintained on antiretroviral therapy (ART) beginning early in childhood [1–3]. While manifested later in life, osteoporosis, which is increased among older adults living with HIV, often has its origins in patterns of bone growth and turnover in childhood and adolescence during which 85–90% of adult peak bone mass is attained [4]. Impaired skeletal growth during these critical periods may compromise bone microarchitecture and peak adult bone mass, which are important determinants of bone strength and fracture risk in later life [5–7]. Optimization of long-term bone health outcomes for those with perinatally acquired HIV is an important area of clinical and public health research particularly for those living in sub-Saharan Africa, where more than 90% of children living with HIV (CLHIV) now reside [8].

A number of cross-sectional studies, conducted mostly in the United States and Europe, observed reduced bone mass among CLHIV [9–13]. Some of the observed difference in bone mineral density may be because of smaller bone volume as statural growth is also adversely affected by HIV [14,15]. We reported lower bone mass among South African CLHIV who initiated ART early in life and had well controlled disease especially among those sustained on ritonavir-boosted lopinavir (LPV/r)-based compared with those switched to efavirenz (EFV)-based regimens [16]. Among adults living with HIV, a number of factors appear to contribute to bone loss including chronic immune activation and systemic inflammation-mediated dysregulation of bone homeostasis favoring bone resorption over formation [17,18]. However, among South African perinatally infected CLHIV, we found both lower bone resorption and lower bone formation with limited evidence of inflammation among those on LPV/r-based as well as EFV-based regimens suggesting that among CLHIV with viral suppression, low bone mass may occur in a setting of overall reduced bone turnover independent of gross inflammation [19].

The goal of this study was to determine if the differences in bone mass and bone turnover observed in our prior cross-sectional study persisted over 2 years of follow-up among South African children with well controlled HIV maintained on LPV/r-based or EFV-based regimens. We also evaluated longitudinal changes of bone mass, bone turnover, inflammatory markers, and the relationships to bone accrual in CLHIV.

Methods

Study population

The CHANGES Bone is a longitudinal cohort study conducted at Rahima Moosa Mother and Child Hospital and Chris Hani Baragwanath Hospital in Johannesburg, South Africa. Recruitment details are presented in previous articles [16,20]. Enrollment began in March 2013 and the study was completed in May 2018. CLHIV were prior participants in a noninferiority randomized clinical trial (RCT) evaluating risk of viral failure comparing initially viral-suppressed children switching to EFV-based therapy with remaining on LPV/r-based therapy [21], and HIV-uninfected controls were recruited from CLHIV's siblings and household members [16]. Children were followed for three visits: enrollment, 12th month, and 24th month. The study was approved by the Institutional Review Boards of Columbia University Irving Medical Center (CUIMC) (New York, New York, USA) and the University of Witwatersrand (Johannesburg, South Africa). Signed informed consent was obtained by each child's parent or guardian; children provided assent if they were at least 7 years old and deemed able to understand.

Measurements and procedures

Anthropometric measurements were obtained with a digital scale and wall-mounted stadiometer [16]. Weight-for-age z score (WAZ), underweight (WAZ <-2), overweight (WAZ >2) for children aged 5–10 years old, height-for-age z score (HAZ), stunted (HAZ <-2) for children aged 5–19 years old and BMI-for-age z score (BAZ) were determined using WHO norms [22]. Height velocity in cm/year was calculated as the difference in height divided by age difference between study visits [23]. Stage of pubertal development was assessed by trained physicians using the highest score of breast or public hair development for female individuals and pubic hair for male individuals according to Tanner Staging method [16]. Duration of physical activity (minutes/week) was estimated from children and caregivers using a validated interviewer-administered recall questionnaire [24]. Those reporting 420 min/week or more of moderate-to-vigorous physical activity met WHO recommendations for muscular fitness and bone health [25].

Among CLHIV, plasma HIV-RNA levels in copies/ml were tested by the Abbott RealTime HIV Assay (Abbott Park, Illinois, USA) and $CD4^+$ cell counts (cells/μl and %) were determined using TruCount Method (BD Biosciences, Germany). ART regimen comparisons were limited to those on a consistent regimen for the entirety of the study.

Bone mass

Whole body and lumbar spine dual-energy X-ray absorptiometry (DXA) scans were performed by licensed radiographers using a single Hologic Discover Wi bone densitometer at the Department of Radiology of Rahima Moosa Mother and Child Hospital. All scans were analyzed by a single technician blinded to HIV status and treatment using Apex software version 3.0 (Hologic Inc, Bedford, Massachusetts, USA) at CUIMC Body

Composition Unit (New York, New York, USA). Whole body and lumbar spine bone area in square centimeters, bone mineral content (BMC) in grams and bone mineral density (BMD) in grams/cm^2 were reported [26]. DXA-derived BMD is a two-dimensional parameter (i.e. areal BMD) and has a number of potentially important limitations in growing children, as it may be strongly influenced by bone size [27]. To address this issue, whole body *z* scores and LS-BMC *z* scores adjusted for age, sex, race and HAZ were calculated using reference norms from the US Bone Mineral Density in Childhood Study [28], as no South African references were available.

Bone turnover markers

Plasma samples were collected at enrollment and at 24 months and stored at −80 °C. Bone formation markers procollagen type I N-terminal propeptide (P1NP) were analyzed for both visits; osteocalcin and sclerostin [29] were analyzed for 24-month visit. Bone resorption markers C-telopeptide of type 1 collagen (CTx) was analyzed for both visits and undercarboxylated osteocalcin (ucOC) [26,30] was analyzed for 24-month visit.

In addition, calciotropic hormones intact parathyroid hormone (iPTH) and 25-hydroxyvitamin D3 (25(OH)D3) concentrations were reported at enrollment. Fibroblast growth factor 23 (FGF-23) was reported at 24-month visit. Those with 25(OH)D3 less than 20 ng/ml were considered having vitamin D insufficiency status [31].

Inflammatory markers

At enrollment and 24-month visit, the following inflammatory markers were measured: interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), monocyte activation marker soluble CD14, and high-sensitivity C-reactive protein (hsCRP).

All plasma assays were conducted at the Biomarkers Core Laboratory, Irving Institute for Clinical and Translational Research, at Columbia University Irving Medical Center (New York, NY, USA).

Statistical analysis

Chi-squared or Fisher exact tests were used to compare proportions. Student *t* tests using means and Wilcoxon rank-sum tests using medians were conducted for normally and nonnormally distributed measurements, respectively. Delta and annual percentage change were reported to measure absolute change and the rate of change over 2 years. Pearson's correlation coefficient was calculated to assess correlations between bone turnover and inflammatory markers.

Linear regression models were built to assess annual percentage change of bone mass comparing CLHIV and controls (or CLHIV on EFV-based and LPV/r-based regimens), adjusted for age, sex, BMI and vitamin D status. Bone mass comparing CLHIV and controls in a subgroup of children who ever had a HAZ greater than 0 were conducted using Student *t* tests. At each visit, linear regression was used to assess bone turnover markers comparing CLHIV and controls (or CLHIV on EFV-based and LPV/r-based regimens), adjusted for age, sex, Tanner stage, height velocity and WB-BA. Similarly, at each visit, linear regression was used to assess inflammation markers comparing CLHIV and controls (or CLHIV on EFV-based and LPV/r-based regimens), adjusted for age, sex, BMI and vitamin D status. At 24-month visit, linear regression was used to assess annual percentage change of bone turnover (or inflammation) markers with annual percentage change of bone mass, adjusted for age, sex, HIV status and enrollment bone turnover (or enrollment inflammation) marks. Next, at each visit, associations between bone turnover and inflammation markers were assessed by linear regression, adjusted for age, sex and HIV status. Generalized Estimating Equation (GEE) models with exchangeable within-subject covariance structure was used to assess longitudinal data association, adjusted for age, sex and HIV status.

Two-tailed *P* values less than 0.05 were considered statistically significant. All statistical analyses were conducted using SAS 9.4 (Cary, North Carolina, USA).

Results

Of 293 CLHIV who completed a prior RCT [21], 220 CLHIV were enrolled. In addition, 220 controls were recruited. Children's demographic characteristics, anthropometric measurements, and physical activity are presented in Tables 1 and 2, and 94.1 and 92.5% completed 12-month and 24-month visits, respectively. CLHIV (49.1% boys) and controls (54.6% boys) with a mean age of 6.4 (±1.3) and 7.0 (±1.5) years old ($P < 0.001$), respectively were enrolled. CLHIV had a higher proportion of stunting throughout the study period. At 12 months and 24 months, pubertal development was more advanced for the controls than CLHIV. Among CLHIV (Table 3**)**, nearly three quarters were virologically suppressed with an HIV RNA less than 40 copies/ml and a CD4$^+$ percent at least 35%, at all three study visits. During the 2 years of observation, 103 children were consistently on EFV and 93 children were consistently on LPV/r. The other antiretroviral drugs included in the regimens were lamivudine combined with either abacavir, stavudine or zidovudine.

Bone mass

As shown in Fig. 1, CLHIV had significantly lower mean WB-BMC, WB-BMD and WB-BMC *z* scores compared with controls at all three study visits. Mean WB-BMC and WB-BMD increased and WB-BMC *z* scores decreased over 2 years among all children. The

Table 1. Characteristics of children living with HIV and controls at enrollment, 12-month, and 24-month visits in Johannesburg, South Africa.

	Enrolment			12-month visit			24-month visit		
Anthropometric measurement	CLHIV ($N=220$)	Control ($N=220$)	P value	CLHIV ($N=214$)	Control ($N=204$)	P value	CLHIV ($N=208$)	Control ($N=200$)	P value
Sex [N (%)]			0.294			0.435			0.429
Male	108 (49.1)	120 (54.6)		107 (50.0)	110 (53.9)		104 (50.0)	108 (54.0)	
Female	112 (50.9)	100 (45.4)		107 (50.0)	94 (46.1)		104 (50.0)	92 (46.0)	
Age at visit (years), mean (SD)	6.38 (1.25)	6.99 (1.53)	<0.001	7.39 (1.26)	8.03 (1.53)	<0.001	8.39 (1.28)	9.04 (1.55)	<0.001
Weight (kg), mean (SD)	19.3 (3.9)	22.4 (5.4)	<0.001	21.6 (4.4)	25.3 (6.6)	<0.001	23.9 (5.2)	28.5 (7.9)	<0.001
Weight for age z score (WHO), mean (SD)	−0.83 (0.92)	−0.29 (1.05)	<0.001	−0.78 (0.93)	−0.21 (1.10)	<0.001	−0.83 (0.95)	−0.15 (1.19)	<0.001
Underweight [N (%)]	24 (10.9)	6 (2.7)	<0.001	20 (9.8)	4 (2.3)	0.003	20 (11.2)	6 (4.3)	0.024
Overweight [N (%)]	36 (16.4)	53 (24.1)	0.057	28 (13.1)	42 (20.6)	0.049	18 (8.7)	44 (22.0)	<0.001
Height (cm), mean (SD)	110.4 (8.3)	116.8 (9.6)	<0.001	116.7 (7.9)	123.5 (9.1)	<0.001	122.7 (7.8)	129.5 (9.2)	<0.001
Height for age z score (WHO), mean (SD)	−1.40 (0.89)	−0.82 (0.91)	<0.001	−1.21 (0.91)	−0.62 (0.95)	<0.001	−1.09 (0.92)	−0.53 (0.89)	<0.001
Height velocity (cm/year), mean (SD)	NA	NA	NA	6.3 (2.0)	6.4 (2.2)	0.627	6.0 (1.6)	6.2 (1.8)	0.236
Stunted [N (%)]	61 (27.7)	19 (8.6)	<0.001	44 (20.6)	12 (5.9)	<0.001	32 (15.4)	9 (4.5)	<0.001
BMI (kg/m^2), mean (SD)	15.7 (1.6)	16.2 (2.1)	0.002	15.7 (1.7)	16.4 (2.5)	0.001	15.7 (1.9)	16.8 (3.1)	<0.001
BMI for age z score (WHO), mean (SD)	0.08 (0.96)	0.28 (1.09)	0.047	−0.04 (0.93)	0.13 (1.17)	0.096	−0.28 (0.96)	0.07 (1.30)	0.002
Tanner stage [N (%)]			0.447			0.012			<0.001
1	218 (99.1)	213 (98.2)		206 (97.6)	182 (91.9)		190 (91.4)	158 (79.0)	
2	2 (0.9)	4 (1.8)		4 (1.9)	16 (8.1)		15 (7.2)	36 (18.0)	
3	0	0		1 (0.5)	0		3 (1.4)	6 (3.0)	

CLHIV, children living with HIV.

mean annual percentage change of WB-BMC (0.15 ± 0.04 vs. 0.14 ± 0.05, $P = 0.254$) and WB-BMD (0.08 ± 0.02 vs. 0.08 ± 0.02, $P = 0.479$) did not differ between CLHIV and controls, even after adjusting for age, sex, BMI and vitamin D status at enrollment. Similarly, LS-BMC and LS-BMD were significantly lower among CLHIV compared with controls and increased over 2 years. Mean annual percentage change of LS-BMC (0.18 ± 0.14 vs. 0.19 ± 0.14, $P = 0.512$) and LS-BMD (0.03 ± 0.04 vs. 0.03 ± 0.04, $P = 0.937$) did not differ between CLHIV and controls, even after adjusting for age, sex, BMI and vitamin D status at enrollment. In the subgroup that ever achieved normal height, CLHIV ($n = 37$) had significantly lower WB-BMC and WB-BMD at three visits, lower WB-BMC z scores and LS-BMC at 24-month visit, and lower LS-BMD at enrollment and 12-month visit compared with controls ($n = 70$).

Among CLHIV, WB-BMC z scores were significantly higher for children switched to EFV compared with those remaining on LPV/r at the three study visits: -0.77 ± 0.77 vs. -1.14 ± 0.78, $P < 0.001$; -0.94 ± 0.74 vs. -1.34 ± 0.70, $P < 0.001$; -1.01 ± 0.76 vs. -1.40 ± 0.69, $P < 0.001$, respectively. Annual percentage change of WB-BMD for CLHIV switched to EFV was higher than children remaining on LPV/r (0.08 ± 0.02 vs. 0.07 ± 0.02, $P < 0.001$) and this remained significant after adjusted for age, sex, BMI and vitamin D status at enrollment.

Bone turnover markers

Univariate analysis results are presented in Table 4. For bone formation markers, mean P1NP was significantly lower among CLHIV than controls at enrollment and 24-month visit, and remained lower after adjusted for age, sex, Tanner stage, height velocity and WB-BA. P1NP

Table 2. Physical activity of children living with HIV and controls at enrollment, 12-month, and 24-month visits in Johannesburg, South Africa.

	Enrolment			12-month visit			24-month visit		
Physical activity (PA)	CLHIV ($N=218$)	Control ($N=220$)	P value	CLHIV ($N=211$)	Control ($N=198$)	P value	CLHIV ($N=208$)	Control ($N=200$)	P value
Moderate and vigorous PA (min), median (IQR)	1265 (645–2120)	1165 (608–2155)	0.844	775 (475–1375)	840 (555–1260)	0.620	810 (490–1233)	825 (423–1285)	0.974
WHO physical activity minutes guidelines category [N (%)]			1.000			0.794			0.551
≥420	182 (83.5)	184 (83.6)		173 (82.0)	165 (83.3)		165 (79.3)	153 (76.5)	
<420	36 (16.5)	36 (16.4)		38 (18.0)	33 (16.7)		43 (20.7)	47 (23.5)	

CLHIV, children living with HIV; IQR, interquartile range.

Table 3. Clinical information of children living with HIV at enrollment, 12-month, and 24-month visits in Johannesburg, South Africa.

Lab test results	Enrollment CLHIV ($N=218$)	12-month visit CLHIV ($N=208$)	24-month visit CLHIV ($N=208$)
Viral load (copies/ml) [*N* (%)]			
TND or LDL ($\leq$20 or $\leq$40)	161 (73.9)	159 (76.4)	158 (76.0)
21–1000	53 (24.3)	37 (17.8)	42 (20.2)
>1000	4 (1.8)	12 (5.8)	8 (3.8)
$CD4^+$ cell count (cells/μl), mean (SD)	1220 (428)	1132 (406)	1061 (344)
$CD4^+$ cell count (cells/μl) [*N* (%)]			
<750	24 (11.1)	28 (13.5)	34 (16.5)
$\geq$750–1000	193 (88.9)	180 (86.4)	172 (83.5)
$CD4^+$ percentage (%), mean (SD)	37.30 (7.07)	37.42 (6.45)	37.56 (6.51)
$CD4^+$ percentage (%) [*N* (%)]			
<25	8 (3.7)	5 (2.4)	6 (2.9)
25–30	25 (11.5)	19 (9.1)	17 (8.3)
30–35	43 (19.8)	51 (24.5)	50 (24.3)
$\geq$35	141 (65.0)	133 (63.9)	133 (64.6)
ART regimen category [*N* (%)]			
EFV-based	111 (50.5)	117 (54.7)	131 (63.0)
LPV/r-based	107 (48.6)	97 (45.3)	77 (37.0)
Other or unknown	2 (0.9)	0	0
ART regimen switch during each two visits [*N* (%)]			
Remained on EFV-based regimen	NA	107 (50.2)	117 (54.7)
Remained on LPV/r-based regimen		96 (45.1)	97 (45.3)
Switched from LPV/r to EFV		10 (4.7)	0
Switched from EFV to LPV/r		0	0

CLHIV, children living with HIV; EFV, efavirenz; LPV/r, ritonavir-boosted lopinavir.

increased over 2 years in both groups and annual percentage change did not differ between CLHIV and controls (0.12 ± 0.26 vs. 0.10 ± 0.20, $P=0.539$). Mean osteocalcin was also lower in CLHIV than controls at 24-month visit. Among CLHIV, none of the bone formation markers were significantly different between those switched to EFV and those remaining on LPV/r.

The bone resorption marker CTx was consistently lower among CLHIV than controls over 2 years in both

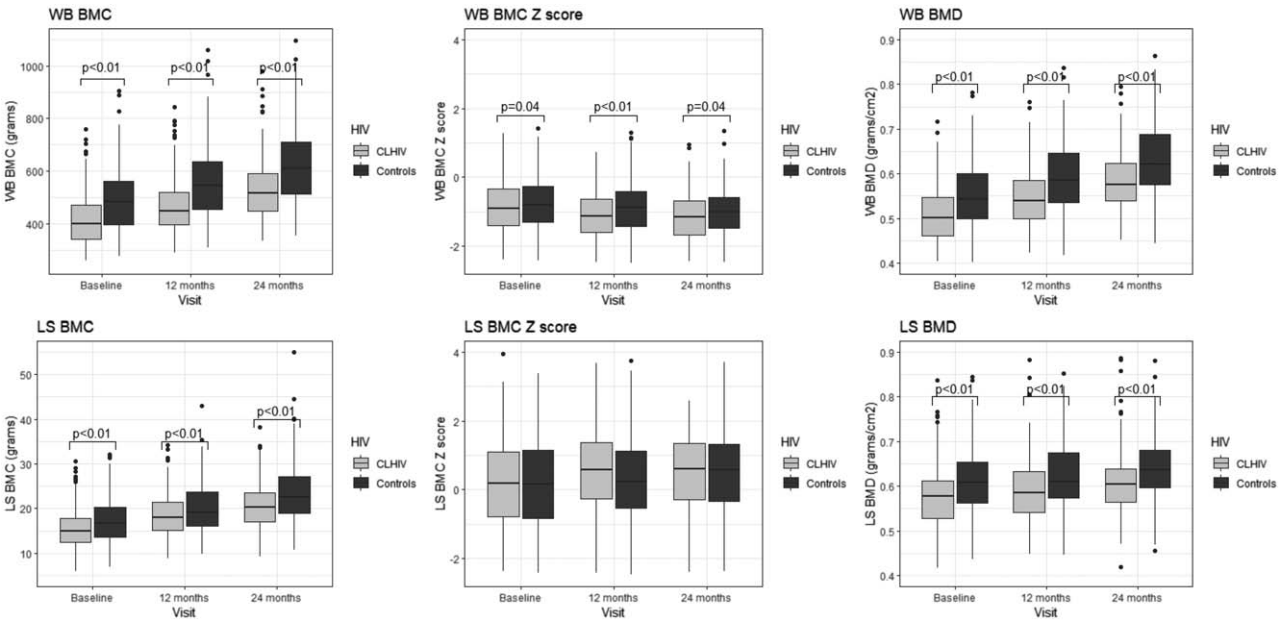

Fig. 1. Whole body and lumbar spine bone mineral content, bone mineral content *z* score and bone mineral density stratified by HIV status at enrollment, 12-month and 24-month visits. BMC, bone mineral content; BMD, bone mineral density; CLHIV, children living with HIV; LS, lumbar spine; WB, whole body.

Table 4. Bone turnover markers stratified by HIV status and antiretroviral therapy regimen at enrollment and 24-month visits.

		Bone turnover markers							
		CLHIV	EFV	LPV/r	Control	*P* value			
Time point	Statistics	*N*=219	*N*=107	*N*=95	*N*=180	HIV + vs. HIV-	EFV vs. LPV/r	EFV vs. HIV-	LPV/r vs. HIV-
P1NP[a] (ng/ml)									
Enrollment	Mean (SD)	583.6 (183.0)	594.7 (188.7)	588.9 (178.3)	634.4 (172.8)	0.005	0.825	0.070	0.041
24-month visit	Mean (SD)	666.0 (192.3)	685.4 (202.7)	652.9 (178.4)	760.0 (265.3)	<0.001	0.238	0.007	0.001
Delta	Mean (SD)	80.4 (211.8)	89.3 (208.5)	61.9 (215.1)	104.9 (243.3)	0.269	0.369	0.552	0.143
CTx[b] (ng/ml)									
Enrollment	Mean (SD)	1.72 (0.63)	1.74 (0.63)	1.76 (0.64)	2.05 (0.69)	<0.001	0.861	<0.001	0.001
24-month visit	Mean (SD)	1.84 (0.68)	1.88 (0.62)	1.79 (0.73)	2.09 (0.76)	0.001	0.385	0.011	0.002
Delta	Mean (SD)	0.12 (0.78)	0.15 (0.71)	0.04 (0.86)	0 (0.81)	0.141	0.349	0.116	0.659
OC[c] (ng/ml)									
24-month visit	Median (IQR)	50.21 (32.52–76.76)	47.02 (29.84–75.53)	55.22 (38.61–81.55)	70.90 (40.85–109.42)	<0.001	0.161	<0.001	0.014
ucOC[d] (ng/ml)									
24-month visit	Median (IQR)	28.62 (8.31–47.48)	26.34 (7.50–48.14)	30.10 (7.84–49.94)	33.06 (9.72–66.06)	0.030	0.683	0.037	0.128
Sclerostin[e] (ng/ml)									
24-month visit	Mean (SD)	0.72 (0.19)	0.72 (0.20)	0.72 (0.18)	0.69 (0.22)	0.211	0.983	0.319	0.300

[a]RIA; Immunodiagnostic Systems, Scottsdale, Arizona, USA.
[b]ELISA; Immunodiagnostic Systems, Scottsdale, Arizona, USA.
[c]ELISA; Immunodiagnostic Systems, Scottsdale, Arizona, USA.
[d]ELISA; Takara Bio Inc, Shiga, Japan.
[e]ELISA; TECOMedical Group, Sissach, Switzerland.

univariate and multivariate analysis adjusting for age, sex, Tanner stage, height velocity and WB-BA. Mean CTx increased over 2 years in both groups and annual percentage change did not differ between CLHIV and controls (0.09 ± 0.29 vs. 0.05 ± 0.24, $P = 0.083$). Mean ucOC was lower in CLHIV than controls at 24 months. There were no significant differences found for CTx and ucOC comparing CLHIV on EFV-based and those on LPV/r-based regimens.

Annual percentage change in P1NP was positively associated with annual percentage change in WB-BMD ($\beta = 0.017 \pm 0.005$, $P = 0.001$) adjusted for age, sex, HIV status and enrollment P1NP. Similarly, annual percentage change in CTx was positively associated with annual percentage change in WB-BMD ($\beta = 0.014 \pm 0.005$, $P = 0.002$) adjusted for age, sex, HIV status and enrollment CTx.

Calciotropic hormones results are presented in Table 5. A higher proportion of CLHIV had vitamin D insufficiency (12.7 vs. 24.7%, $P = 0.002$) at enrollment.

Inflammatory markers

Results of inflammatory markers are presented in Table 6. TNF-alpha was consistently lower whereas soluble CD14 and hsCRP remained higher among CLHIV than controls, at enrollment and at 24 months. These findings remained after adjusting for age, sex, BMI and enrollment vitamin D status. Over 2 years, IL-6 and hsCRP increased

Table 5. Calciotropic hormones stratified by HIV status and antiretroviral therapy regimen at enrollment and 24-month visits.

			Calciotropic hormones							
			CLHIV	EFV	LPV/r	Control	*P* value			
	Time point	Statistics	*N*=219	*N*=107	*N*=95	*N*=180	HIV + vs. HIV−	EFV vs. LPV/r	EFV vs. HIV−	LPV/r vs. HIV−
iPTH[a] (pg/ml)	Enrollment	Mean (SD)	31.1 (12.9)	31.34 (13.45)	30.47 (12.29)	32.1 (15.7)	0.509	0.637	0.692	0.358
25 (OH)D3[b] (ng/ml)	Enrollment	Mean (SD)	30.59 (9.78)	26.43 (7.52)	35.02 (8.95)	24.29 (6.33)	<0.001	<0.001	0.016	<0.001
Vitamin D insufficiency	Enrollment	*N* (%)	27 (12.7)	20 (19.4)	4 (4.3)	44 (24.7)	0.002	0.002	0.305	<0.001
FGF-23[c] (RU/ml)	24-month visit	Median (IQR)	80.86 (61.38–112.14)	83.04 (59.82–98.72)	76.04 (61.53–114.3)	71.76 (58.02–96.40)	0.009	0.274	0.007	0.235

[a]RIA; Scantibodies Laboratory, Santee, California, USA.
[b]LCMS; Agilent, Santa Clara, California, USA.
[c]ELISA; Immutopics, San Clemente, California, USA.

Table 6. Inflammatory markers stratified by HIV status and antiretroviral therapy regimen at enrollment and 24-month visits.

Inflammatory markers											
			CLHIV	EFV	LPV/r	Control	*P* value				
	Time point	Statistics	$N=219$	$N=107$	$N=95$	$N=180$	HIV + vs. HIV−	EFV vs. LPV/r	EFV vs. HIV−	LPV/r vs. HIV−
IL-6[a] (pg/ml)	Enrollment	Median (IQR)	0.90 (0.58–1.43)	0.80 (0.54–1.31)	0.96 (0.61–1.61)	0.88 (0.54–1.56)	0.889	0.038	0.180	0.384
	24-month visit	Median (IQR)	1.25 (0.76–2.11)	1.03 (0.68–1.80)	1.56 (0.89–2.19)	1.12 (0.77–1.86)	0.379	0.012	0.317	0.026
	Delta	Median (IQR)	0.31 (−0.32 to 1.09)	0.30 (−0.25 to 0.88)	0.41 (−0.39 to 1.40)	0.19 (−0.41 to0.79)	0.156	0.648	0.248	0.130
TNF-alpha[b] (pg/ml)	Enrollment	Median (IQR)	1.87 (1.48–2.42)	1.59 (1.24–2.18)	2.13 (1.73–2.75)	2.36 (1.93–2.89)	<0.001	<0.001	<0.001	0.095
	24-month visit	Median (IQR)	1.76 (1.53–2.13)	1.68 (1.45–1.96)	1.91 (1.63–2.33)	2.13 (1.82–2.45)	<0.001	<0.001	<0.001	0.007
	Delta	Median (IQR)	−0.08 (−0.64 to 0.40)	0.11 (−0.33 to 0.44)	−0.17 (−0.83 to 0.29)	−0.28 (−0.94 to 0.28)	0.025	0.017	0.001	0.553
hsCRP[c] (mg/dl)	Enrollment	Median (IQR)	0.77 (0.30–2.39)	0.79 (0.33–2.24)	0.59 (0.30–1.62)	0.38 (0.30–1.34)	<0.001	0.136	0.001	0.132
	24-month visit	Median (IQR)	0.58 (0.29–1.95)	0.52 (0.29–1.92)	0.60 (0.29–1.59)	0.30 (0.29–0.96)	<0.001	0.939	0.006	0.012
	Delta	Median (IQR)	−0.01 (−1.04 to 0.59)	−0.02 (−0.98, 0.53)	−0.01 (−0.74 to 0.59)	−0.01 (−0.25 to 0.32)	0.169	0.565	0.184	0.657
Soluble CD14[d] (ng/ml)	Enrollment	Median (IQR)	1323.5 (1108.0–1669.0)	1362.5 (1146.0–1652.0)	1271.0 (1087.0–1725.0)	1102.5 (892.6–1344.0)	<0.001	0.548	<0.001	<0.001
	24-month visit	Median (IQR)	1980.7 (1573.9–2357.8)	2015.5 (1614.5–2379.9)	1939.9 (1581.2–2351.7)	1311.0 (1060.3–1712.6)	<0.001	0.577	<0.001	<0.001
	Delta	Median (IQR)	567.2 (189.4–965.7)	633.5 (215.1–965.7)	448.0 (137.1–947.0)	193.6 (−146.9 to 685.2)	<0.001	0.503	<0.001	0.001

CLHIV, children living with HIV; EFV, efavirenz; IQR, interquartile range; LPV/r, ritonavir-boosted lopinavir.
[a]ELISA; R&D Systems, Minneapolis, Minnesota, USA.
[b]ELISA; R&D Systems, Minneapolis Minnesota, USA.
[c]Cobas Integra 400 Plus; Roche Diagnostics, Indianapolis, Indiana, USA.
[d]ELISA; R&D Systems, Minneapolis, Minnesota, USA.

at a similar rate among the two groups. Annual percentage change in TNF alpha was positive among CLHIV and negative among controls (0.03 ± 0.27 vs. -0.03 ± 0.18, $P = 0.006$) and annual percentage change in soluble CD14 was greater among CLHIV than controls (0.25 ± 0.30 vs. 0.16 ± 0.29, $P = 0.004$).

Among CLHIV, IL-6 and TNF-alpha were higher at enrollment as well as at 24 months among children remaining on LPV/r than those switched to EFV. Measured by annual percentage change, TNF-alpha was stable among children remaining on LPV/r and slightly increased among those switched to EFV (0 ± 0.27 vs. 0.08 ± 0.27, $P = 0.049$).

In further analysis, soluble CD14 was negatively correlated with WB-BMC z scores at enrollment (Pearson correlation $r = -0.102$, $P = 0.042$). At 24 months, IL-6 was negatively correlated with WB-BMC z scores (Pearson correlation $r = -0.107$, $P = 0.031$) and soluble CD14 was negatively correlated with WB-BMC z scores (Pearson correlation $r = -0.125$, $P = 0.012$). Over 2 years, soluble CD14 annual percentage change was negatively associated with WB-BMC annual percentage change ($\beta = -0.017 \pm 0.008$, $P = 0.034$), after adjusted for age, sex, HIV status and enrollment soluble CD14.

Relationships between bone turnover markers and inflammatory markers

We assessed the relationships between inflammatory and bone turnover markers. Among all children, IL-6 was negatively associated with P1NP at enrollment. TNF-alpha was positively associated with all bone formation markers: P1NP, osteocalcin and sclerostin at 24 months, even after adjusted for age, sex and HIV status. TNF-alpha was also positively associated with bone resorption marker CTx at 24 months. Soluble CD14 was negatively related with osteocalcin at 24 months and hsCRP was negatively related with P1NP at both visits.

Assessed by GEE models, IL-6 remained negatively associated with P1NP longitudinally for all children, even adjusted for age, sex and HIV status. The negative association between hsCRP and P1NP remained among both groups after adjusting for age, sex and HIV status or ART regimen for CLHIV.

Discussion

To our knowledge, this is the first longitudinal study conducted among CLHIV in sub-Saharan Africa to include comprehensive evaluation of bone mass, as well as of bone turnover and inflammation. In addition to factors specific to chronic HIV infection, the higher prevalence of malnutrition and the distinct patterns of childhood

infectious diseases found among children living in South Africa may be expected to adversely affect skeletal health [32–36]. This study, which had well matched uninfected controls and high retention and assessed bone mass adjusted for potential confounders with over 2 years of observation, found that South African CLHIV on a stable ART regimen had persistently lower bone mass with lower bone formation and resorption than controls.

We previously reported in this same cohort, lower bone mass of CLHIV who had been on ART for 2.8–8.7 years and achieved viral suppression early in life [16]. In this study, the rate of increase in bone mass in CLHIV was similar to controls. CLHIV failed to 'catch up' in bone accrual and to achieve bone mass comparable with their uninfected peers. The static nature of the deficits in bone we observed are similar to findings in a small 10-year prospective cohort of Italian HIV-infected children who switched to TDF-containing regimens [37,38]. Several studies of young adults with HIV have also reported lower BMD than controls [12,39–41]. Taken together, these findings suggest that bone mass deficits occur early and persist throughout childhood.

In this study, we find further evidence that continued exposure to LPV/r-containing regimens exerts a greater adverse effect on bone development than switching to EFV-based ART [16,42], providing further evidence that this agent may be less desirable than some alternatives. Adverse effects of protease inhibitors or nonnucleoside reverse transcriptase inhibitors (NNRTI), such as EFV on bone quality and osteoblasts are reported in both animal and human studies [43–46]. Greater bone loss has been observed among adults living with HIV on protease inhibitor-containing regimens than those receiving NNRTI-containing regimens but findings vary depending on the specific agents involved [47–49]. A major strength of our study was the ability to adjust regimen comparisons for potential confounders, including age, sex, anthropometric measurements and vitamin D intake. As all children in the follow-up study were randomly assigned to remain on LPV/r or switch to an EFV-based regimen following viral suppression, the higher rate of bone mass increase we observed among those on EFV-based regimens suggests there is potential for at least partial recovery of bone accrual when more 'bone-friendly' regimens are used. The impact on bone development of newer agents including nucleotide analog reverse transcriptase inhibitors and integrase strand transfer inhibitors (INSTI) that are being used more widely in children and adolescents warrants clinical as well as in-vitro study.

As histomorphologic studies require bone biopsy, we instead used serum biomarkers to investigate bone remodeling processes involved in reduced bone accrual [10,11,14,50–52]. We found that bone formation and bone resorption were both consistently lower among CLHIV compared with uninfected controls and increased with similar rates of change over 2 years. This is in contrast to an older study that reported increased bone formation (measured by serum P1NP and bone-specific alkaline phosphatase, BALP) and bone resorption (measured by urine N-terminal telopeptide of type I collagen, NTx) among white CLHIV aged 6–17 years receiving protease inhibitor-based therapy (indinavir, nelfinavir and ritonavir) compared with controls [14,52], which persisted after 1 year of follow-up on older ART regimens [10]. Others have reported elevated bone turnover markers and a decreased trend with 4 years follow-up among young adults with median age 24 years [53]. Of note, these longitudinal studies [10,53] found a pattern of normalization of bone homeostasis among CLHIV, adolescents and young adults, meaning that the bone turnover activity approached values observed in uninfected individuals over time.

Consistent with our prior cross-sectional study [19], overall. we did not detect elevated pro-inflammatory markers in CLHIV over the 2-year study period compared with controls. This may be because of well controlled viral replication and long-term ART in our study cohort. A study of adolescents, which stratified by detectable and undetectable viral load status, found adolescents with detectable viral load had significantly lower IL-6 levels than controls, and those with an undetectable viral load had IL-6 levels comparable with controls [54]. In other studies, comparing uninfected controls with CLHIV initiating or changing ART who were followed for 48 weeks, IL-6 and TNF-alpha have been reported to decrease 48 weeks after ART initiation in one [55] but not another where comparable levels of IL-6 and TNF-alpha were observed [56]. In a third study, comparing CLHIV on protease inhibitor-based or NNRTI-based ART and controls aged 2–21 years old, no differences in IL-6 and TNF-alpha were observed [57].

The elevated soluble CD14 among CLHIV that we observed in our cohort has also been observed in other studies of perinatally infected children, adolescents, youth and adults living with HIV, regardless of virologic suppression and ART status [58–61]. Consistent with findings from a study among male adolescents living with HIV [61], we found an inverse correlation between soluble CD14 and bone mass (WB-BMC z scores) at enrollment and at 24 months and the rate of increase in soluble CD14 also negatively correlated with rate of increase in bone mass (WB-BMC). The authors suggest that elevated soluble CD14, a marker of macrophage activation, increases osteoclast activity leading to increased bone resorption [61]. In our study, we did not observe a significant association between soluble CD14 and bone resorption markers. We did find a consistent inverse correlation between hsCRP and the

bone formation biomarker P1NP. Further studies are needed to understand possible bone remodeling processes mediated by soluble CD14 in the setting of chronic infection with HIV.

To our knowledge, this is among the first studies to examine pro-inflammatory cytokines in South African CLHIV on different ART regimens. We found higher levels of IL-6 and TNF-alpha among CLHIV on protease inhibitor-based than those on NNRTI-based regimen over a 2-year follow-up period. Similarly, higher IL-6 has been reported among adults treated with protease inhibitor-based compared with NNRTI-based regimen [62]. A trial that randomly assigned participants to protease inhibitor-based and NNRTI-based ART found greater decrease in IL-6 and hsCPR in CLHIV on NNRTI-based than protease inhibitor-based ART over 4 years follow-up [63]. We did not observe the same longitudinal trends in those markers but we did find persistently higher TNF-alpha among CLHIV on protease inhibitor-based regimen. How these findings help us to identify 'inflammation-reducing' treatments are unclear, and further studies for better understanding of the clinical implications of different inflammatory signatures during ART are also warranted.

In conclusion, our study suggests that the deficits in BMD and decreased bone turnover among CLHIV are established early in life, and persist despite effective ART and when available use of alternatives to LPV-based regimens may allow for better bone accrual. Evaluation of bone development during high growth periods of childhood and adolescence are warranted in studies of new and emerging antiretroviral agents in order to better inform treatment decision-making and clinical guidelines.

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M.B. and F.P. recruited participants, obtained consents, and performed clinical assessment in South Africa;

S.S., C.T.J. and Y.S. communicated, collected and cleaned data; G.D. and B.R. performed DXA scans reading;

S.S., Y.S. conducted data analysis; S.M.A., M.T.Y., S.S., Y.S. led the manuscript-writing efforts with all co-authors contributing to subsequent drafts and the final version of the manuscript.

Conflicts of interest

There are no conflicts of interest.

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