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Longitudinal Changes of Bone Mineral Density and Metabolism in Antiretroviral-Treated Human Immunodeficiency Virus-Infected Children

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Highly active antiretroviral therapy (HAART) may be a contributory factor for a decreased bone mass and altered bone metabolism in HIV-infected children. However, the evolution of bone mineral density (BMD) and bone metabolism during HAART has not been studied yet. In the current longitudinal study we monitored the changes of BMD and bone metabolism over a period of 12 months. Thirty-two HIV-infected children (15 girls and 17 boys), aged from 6.3 to 17.7 yr, with a long duration of HAART exposure (40.0 months at baseline) were enrolled in the study. As a control group, 381 healthy volunteers of comparable age were assessed. BMD was measured at the lumbar spine and whole skeleton by dual-energy x-ray absorptiometry. Bone-specific alkaline phosphatase (BALP, as bone formation index) and N-terminal telopeptide of type I collagen (as bone resorption index) were measured in serum and urine, respectively. BMD values at baseline were significantly lower at all skeletal sites than those of control subjects. The annual increment of spine BMD was comparable to normal, whereas that of the whole skeleton was significantly lower ($P < 0.04$). BALP and N-terminal telopeptide of type I collagen concentrations were significantly higher compared with controls at baseline and at follow-up. BALP annual changes of HIV patients were significantly different from normal. Our data confirm the presence of low BMD and bone metabolism derangement in HIV-infected children treated with HAART. The role of possible therapeutic approach to restore bone mass and metabolism should be assessed in pediatrics. (*J Clin Endocrinol Metab* 89: 24–28, 2004)

THE USE OF highly active antiretroviral therapy (HAART) has consistently decreased morbidity and mortality rates of HIV-infected patients (1). However, HAART has been associated with the development of several acute and chronic complications (2–4). Osteopenia, osteoporosis, and impairments of bone metabolism have been recently reported as frequent findings in HAART-treated patients (5–9). To date, no definitive cause for bone mass and bone metabolism alterations has been found. Variable associations with the use of protease inhibitors (PIs), development of lipodystrophy, nutritional and hormonal factors, and HIV infection *per se* have been proposed (5, 10, 11).

Reduced bone mineral density (BMD) has also been found in HIV-infected children receiving antiretroviral treatment (12–15). This observation is of great concern because of the dramatic improvement in life expectancy of HIV-infected children. Childhood and adolescence are in fact crucial periods of life for the attainment of an optimal bone mass. Bone mass changes markedly during childhood and adolescence, to reach a peak in the third decade of life (16, 17), and peak bone mass is a major determinant for the development of osteoporosis later in life. Therefore, impairments in obtaining an optimal bone mass should be identified during childhood to avoid future complications.

Bone metabolism rate can be precisely assessed by histomorphometry of the iliac crest, which represents the gold standard for estimating the status of bone turnover (18). However, bone biopsy is an invasive procedure that is not feasible for routine use in the evaluation of bone metabolism. More readily available to physicians and researchers are biochemical tests performed on blood or urine samples, which mirror the ongoing bone metabolic processes (19). These biochemical markers are based on the measurements of either an enzymatic activity characteristic of the bone-forming or -resorbing cells or bone matrix components released into the circulation during bone apposition or resorption. The concentration of bone metabolism markers changes markedly during childhood and adolescence. Maximum levels are observed in infancy and during the pubertal period when skeletal growth is more rapid (20–22). HAART-treated children and adolescents show remarkable alterations of bone metabolism rate, assessed by biochemical markers of bone turnover (12–14).

The evolution of bone mineralization and metabolism over time in HAART-treated patients has been scarcely studied. Few studies reporting conflicting results were performed in adults (23–25), but no data are available for HIV-infected youths. The lack of knowledge about the changes that occur over time prompted us to study longitudinally a group of vertically HIV-infected children and adolescents treated with HAART, and to compare the measurements with those obtained in a large control population.

Abbreviations: BALP, Bone-specific alkaline phosphatase; BCE, bone collagen equivalents; BMD, bone mineral density; CV, coefficient of variation; HAART, highly active antiretroviral therapy; PI, protease inhibitor; sBMD, spinal BMD.

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Subjects and Methods

Subjects

Eligible for the present study were HIV-infected Caucasian children and adolescents who were receiving a protease inhibitor (PI)-based HAART regimen and who were followed at the Pediatric Unit of L. Sacco Hospital in Milan. Forty patients agreed to participate in the study, but only 37 completed the 1-yr follow-up. One patient was excluded because of African descent and four because they did not receive antiviral treatment. We therefore studied 32 patients (15 girls and 17 boys), aged from 6.3 to 17.7 yr at baseline (Table 1). None of the subjects had clinically apparent renal, cardiac, or intercurrent disease at the time of evaluation. None of the children had previously received or were currently treated with sex hormones, corticosteroids, vitamin D, or calcium preparations. All 32 HIV patients were receiving antiretroviral treatment with d4T, 3TC, and one PI (25 patients received indinavir, five received ritonavir, and two received nelfinavir). All patients had a long-lasting immune recovery and control of viral replication (Table 1).

As a control group, we studied 381 healthy volunteers (172 girls and 209 boys) of comparable age (5.7 to 19.2 yr). All subjects were healthy and appropriately physically active for their age; none was involved in competitive sport activities. Their mean height and weight measurements were 146.9 (0.9) cm and 42.0 (0.8) kg, respectively. Candidates were excluded if their anthropometric measurements were not within the 3rd and 97th centiles for age (26), if they had a history of chronic illness, if they had one or more fractures, and if they had taken any medication, hormone, vitamin preparation, or calcium supplements regularly.

All candidates for this study underwent physical examination to obtain anthropometric measures and to assess pubertal development. Body weight was measured to the nearest 0.1 kg on a balance beam scale (Seca, Hamburg, Germany), and height was measured to the nearest millimeter using a wall-mounted stadiometer (Holtain Ltd., Crosswell, UK). Pubertal stage was defined according to Tanner criteria (27). None of the children had delayed puberty. The ages of the HIV-infected girls at Tanner stages II, III, IV, and V (10.3, 11.9, 14.0, and 15.9 yr, respectively) were similar to those of the healthy girls (10.7, 12.3, 14.3, and 16.7 yr). Similarly, the ages of HIV-infected boys at Tanner stages II, III, IV, and V (11.7, 13.2, 14.6, and 16.8 yr, respectively) did not differ from those of control boys (12.4, 13.7, 15.1, 17.6 yr).

TABLE 1. Characteristics of 32 vertically HIV-infected children at baseline and after 12 months of additional exposure to HAART

	Baseline	After 12 months
Age (yr)	12.4 (0.5)	13.4 (0.5)
Weight (kg)	40.8 (2.4)	44.8 (2.3)
(Z-score)	−0.77 (0.19)	−0.71 (0.18)
Height (cm)	146.9 (2.9)	153.1 (15.8)
(Z-score)	−0.65 (0.22)	−0.48 (0.17)
Pubertal development (Tanner stage)	I = 8 II = 10 III = 4 IV = 1 V = 9	I = 5 II = 3 III = 5 IV = 4 V = 15
CDC clinical stage	A = 11 B = 9 C = 12	
CDC immunological stage	1 = 7 2 = 14 3 = 11	
Previous zidovudine/zidovudine + didanosine exposure (months)	41.0 (5.1)	
Stavudine + lamuvidine + PI exposure (months)	40.0 (0.7)	52.3 (0.7)
$CD4^+$ cells ($\times 10^6$ cells/liter)	986.0 (81.2)	965.3 (63.3)
$CD4^+$ cells (%)	31.6 (1.8)	34.0 (1.5)
Children with HIV-RNA < 50 copies/ml	32	32

Data are expressed as mean (SE).

Informed consent was obtained from all the parents or legal guardians of all patients and volunteers. The study was made in accordance to the Declaration of Helsinki, and the Ethical Committee of L. Sacco Hospital approved it.

Study protocol

Bone mineral measurements and assessment of biochemical markers of bone turnover were obtained at baseline and after about 1 yr [12.5 (0.1) months]. Control subjects were studied only once for ethical reasons.

Bone mineral measurements

BMD was measured at the L2–L4 vertebrae level and in the whole skeleton. Longitudinal data indicate a differential growth pattern of arms and legs in HAART-treated children (28). Therefore, BMD values of arms and legs were obtained from the whole body scan. The data were analyzed with proper pediatric software (version 1.5h, Lunar Corp., Madison, WI). BMD measurements were made with a dual-energy x-ray absorptiometer (DPX-L, Lunar Radiation Corp., Madison, WI). The instrument was calibrated on a daily basis according to the manufacturer's instructions. Reproducibility was calculated as coefficient of variation (CV) obtained by weekly measurements of a standard phantom on the instrument and by repeated measurements obtained in three children of different ages. The CV of our instrument is 0.6% with the standard phantom; *in vivo* we calculated a CV of 1.4% for the lumbar spine and 1.5% for the whole skeleton. According to published data, the effective radiation dose for each scan is about 0.3 μSv for the lumbar spine and less than 0.03 μSv for the whole body scans (29).

Biochemical measurements

Blood was allowed to clot immediately after venipuncture; serum was separated by centrifugation, and it was stored at −30 C until analysis. Urine specimens were collected between 1000 and 1200 h as the second voiding of the day, to minimize the effect of circadian rhythm of excretion of collagen degradation products (21). Samples were aliquoted immediately and stored at −30 C until analysis.

Bone-specific alkaline phosphatase (BALP) was measured in serum as a bone formation marker, using a commercial immunoassay (Metra BAP, Quidel Corp., San Diego, CA). Intraassay reproducibility was less than 4%, and interassay variation was less than 7%. Sensitivity was 0.7 U/liter.

We measured urine concentration of N-terminal telopeptide of type I collagen (NTx) as a bone resorption index. NTx was measured using an enzyme-immunosorbent assay (Osteomark, Ostex, Seattle, WA). Assay values were standardized to an equivalent amount of bone collagen and were expressed in nanomoles bone collagen equivalents (BCE) per liter (nmol BCE/liter). The sample results from a single urine collection were normalized for urine dilution by urine creatinine analysis and were reported as nanomoles BCE/millimoles creatinine. In our laboratory, the intraassay variation was less than 10%. The interassay precision was less than 9%, and sensitivity was 20 nmol BCE/liter.

Urine creatinine was measured by a standard automated method.

The determination of HIV-1 copy numbers was carried out using a quantitative chain reaction assay (Amplicor HIV Monitor, Roche Diagnostic Systems, Basel, Switzerland) according to the manufacturer's instructions.

Statistical analysis

Descriptive statistics were calculated for all the variables, and data are expressed as the mean (SE), unless otherwise stated. All statistical analyses were conducted at the $\alpha = 0.05$ level and were two-tailed. Distribution of the variables were checked using the Shapiro-Wilk W test. The statistical software JMP IN (SAS Institute, Inc., Cary, NC) was used for the analyses.

Multivariate analyses were performed to evaluate the differences between HIV+ patients and control subjects, after controlling for confounding variables. Bone metabolism indices or bone mineral measurements were the dependent variables, whereas sex, age, Tanner stage, and anthropometric measurements were the confounding variables, and presence of HIV+ disease was the independent dichotomous variable.

When analyzing BMD variables, bone area was included in the multivariate model. All anthropometric measurements were initially included, and the backward procedure was used to build the best model.

Changes of BMD and bone metabolism indices that occurred during the year of follow-up have been compared with those estimated for healthy children and calculated in the control group as previously described (30). Briefly, slopes for age changes were obtained using regression analyses, with age as the independent variable and BMD or bone metabolism markers as the dependent variable. Estimated values were then calculated for baseline and follow-up measurements. The difference between the two estimates was then compared with that observed during follow-up using paired *t* test. The equations used to obtain estimated values of BMD were:

$$\text{sBMD} = 0.720 - 0.030\ \text{age} + 0.00326\ \text{age}^2$$

$$\text{TBBMD} = 0.943 - 0.026\ \text{age} + 0.00234\ \text{age}^2$$

$$\text{Arms BMD} = 0.547 - 0.00583\ \text{age} + 0.00145\ \text{age}^2$$

$$\text{Legs BMD} = 0.706 - 0.00094\ \text{age} + 0.00207\ \text{age}^2$$

The equations used to obtain estimated values of bone metabolism markers were:

$$\text{BALP} = -343.01 + 127.75\ \text{age} - 10.4\ \text{age}^2 + 0.248\ \text{age}^3$$

$$\text{NTx} = -725.34 + 264.26\ \text{age} - 20.98\ \text{age}^2 + 0.48\ \text{age}^3$$

Patients were grouped according to the clinical and immunological stage of disease. Comparisons between the groups have been conducted using multivariate analyses to correct for sex, age, and anthropometric differences.

Results

Bone mineral measurements

The values of bone mineral measurements of HIV patients are shown in Table 2. Lumbar spine and whole skeleton BMD values of control subjects were 0.869 (0.018) and 0.996 (0.012) g/cm^2, respectively, whereas BMD values of arms and legs were 0.707 (0.011) and 1.021 (0.019) g/cm^2, respectively.

Multivariate models for the comparison between the two groups included sex, age, Tanner stage, and weight as the confounding variables. Spine BMD measurements of HIV patients were significantly lower compared with control subjects at baseline ($\beta = 0.049$; $P < 0.0001$) and at follow-up ($\beta = 0.055$; $P < 0.0001$). Similarly, BMD values of the whole skeleton were lower in HIV patients at baseline ($\beta = 0.045$; $P < 0.0001$) and after 1 yr ($\beta = 0.061$; $P < 0.0001$). BMD values of the arms at baseline were significantly lower in the patents' group ($\beta = 0.034$; $P < 0.0001$); after 1 yr of follow-up, the difference between the two groups was greater and still significant ($\beta = 0.052$; $P < 0.0001$). HIV patients showed markedly lower BMD values of the legs compared with control subjects both at baseline ($\beta = 0.044$; $P < 0.0001$) and at follow-up ($\beta = 0.062$; $P < 0.0001$).

During the follow-up period, spinal BMD (sBMD) of HIV patients increased on average by 0.069 (0.01) g/cm^2. The increment observed was not statistically different (t = 1.3; $P = 0.17$) from the sBMD increase expected for healthy children [0.055 (0.003) g/cm^2]. Whole body BMD of HIV patients increased by 0.016 (0.007) g/cm^2 over 12 months. The observed increment was significantly different (t = −2.8; $P = 0.0038$) from that estimated for healthy children [0.035 (0.002) g/cm^2]. The annual BMD increment of the arms observed in our patients was 0.014 (0.004) g/cm^2, whereas the increment estimated for healthy children was 0.032 (0.001) g/cm^2. The difference was highly significant (t = −4.0; $P = 0.0004$). The BMD increment of the legs of HIV patients [0.050 (0.013) g/cm^2] did not differ from the estimated one [0.054 (0.002) g/cm^2; t = −0.23; $P = 0.81$].

Analyses comparing subjects grouped according to the clinical stage of the disease and the degree of immunodepletion did not show differences in BMD measurements.

TABLE 2. Bone mineral measurements and bone metabolism indexes of 32 vertically HIV-infected children at baseline and after 12 months of additional exposure to highly active antiretroviral therapy

	Baseline	After 12 months
Lumbar spine BMD (g/cm^2)	0.803 (0.034)	0.875 (0.034)
Total body BMD (g/cm^2)	0.913 (0.020)	0.933 (0.022)
Arms BMD (g/cm^2)	0.647 (0.016)	0.665 (0.016)
Legs BMD (g/cm^2)	0.923 (0.035)	0.980 (0.036)
BALP (U/liter)	124.1 (9.0)	127.2 (9.1)
NTx (nmol BCE/mmol creatinine)	454.8 (49.9)	329.2 (36.1)

Data are expressed as mean (SE).

Biochemical measurement

Serum BALP and urine NTx concentrations measured at baseline and after 12 months are shown in Table 2. Mean BALP levels of control children were 105.3 (6.0) U/liter, whereas mean NTx concentration was 230.5 (11.6) nmol BCE/mmol creatinine.

Multivariate models for BALP included sex, age, Tanner stage, and anthropometric measurements as confounding variables. BALP serum levels of HIV patients were significantly higher compared with controls at baseline [$\beta = 17.9$ (5.2); $P = 0.0008$]. After 1 more year of HAART, the BALP serum levels were still higher than those of control subjects [$\beta = 20.6$ (5.2); $P = 0.0002$].

The multiple regression models for the analyses of NTx were similar to the former, and included sex, age, weight, and height as confounding variables. At baseline HIV patients had NTx values significantly higher than those of healthy children [$\beta = 97.7$ (13.6); $P < 0.0001$]. The difference between the two groups was reduced after 1 yr [$\beta = 79.8$ (15.0)], but still significant ($P < 0.0001$).

The mean difference of BALP serum levels observed in HIV patients was 2.8 (6.4) U/liter, significantly different ($P = 0.033$) from that estimated for healthy children [−11.1 (1.9) U/liter]. The NTx urine measurements of HIV children showed a net mean decrease [−81.1 (45.3) nmol BCE/mmol creatinine]. However, the observed value was not statistically different ($P = 0.27$) from that estimated for control subjects [−26.6 (4.5) nmol BCE/mmol creatinine].

Discussion

Bone density and bone metabolism changes during HAART treatment in HIV-infected children are largely unknown. The present study was designed to monitor vertically HIV-infected children in a short-term follow-up study. BMD was measured at baseline and after 1 yr at lumbar spine and whole skeleton by dual-energy x-ray absorptiometry. Bone mass measurements obtained with dual-energy x-ray absorptiometry are largely influenced by the size of the bone

(31). This is particularly important when changes occur over time, as in children and adolescents who increase bone mass and bone size. For this reason, several methods have been proposed to overcome the problem (32). In the current study, we used multivariate analyses to compare the bone mineral measurements of HIV patients and control subjects and to control for the confounding effect of sex, age, pubertal development, and anthropometric measurements (33). After correction of confounding variables, we found that BMD values were markedly lower at both sites compared with healthy children. This finding confirms previous reports that showed significantly decreased values of bone density in perinatally HIV-infected girls (12), in prepubertal children (15), and in HAART-treated children and adolescents (13).

Longitudinal surveys performed in adult patients showed a variable degree of improvement of bone mineral measurements during HAART (24, 25). These data suggest that the amount of BMD gained depends on $CD4^+$ cells increase and HIV suppression, although the rate of increase of BMD might be slow. The BMD values of our patients increased compared with baseline after an additional year of HAART, but they remained significantly lower than those of control subjects. To better define the evolution over time of BMD in our patients, we estimated from our control population the increases of BMD that would occur in healthy children, and we compared them with those observed in HIV patients. The analyses showed that BMD measured at the lumbar spine increased at a rate comparable to that estimated for healthy children and adolescents. Conversely, whole skeleton BMD increased at a much lower rate. Moreover, analyses of the evolution of bone density of the limbs showed that BMD increments of the legs were comparable to the ones estimated for healthy children. On the contrary, BMD values of the arms changed at a much lower rate than expected. Although the design of the study does not allow us to determine exactly the role of HAART in determining low bone mass, our data indicate that HAART treatment in children does not lead to a short time recovery of BMD, as seen in adult patients. Moreover, these results suggest a differential effect of treatment on bone mineral accretion. Weight-bearing sites (axial skeleton, legs) grow at a normal rate, whereas non-weight bearing sites show a slower growth rate. The reasons for this observation are unclear. We speculate that the beneficial effect of weight on bone mineral accretion might counteract a deleterious effect of treatment on the skeleton. However, longer follow-up studies are needed to confirm these findings.

There is evidence for alterations of bone metabolism in adult (6, 25, 34) and young (13, 14) HAART-treated HIV patients. However, data from adult patients are discordant, showing either low bone formation rate (6, 34) or elevated serum levels of bone formation markers (25, 34). Similarly, bone resorption was found to be enhanced (25, 34), or normal (6). Findings for HIV youth are somehow different. Bone formation indexes (osteocalcin, BALP, propeptide of type I procollagen) have been found to be very elevated in HAART-treated young patients (13, 14). Moreover, bone resorption rate has been found to be higher than normal (13). In the present study we found elevated measurements of BALP and NTx at baseline and after an additional year of treatment. Moreover, the data on the annual changes showed that both indexes decrease at a lower (BALP) or similar (NTx) rate compared with estimated values obtained from the control population. These data confirm and extend previous observations and indicate that bone metabolism alterations do not improve over time during HAART in HIV children and adolescents. High bone metabolism rate could explain low bone density values, because high bone turnover rate is associated with osteopenia and osteoporosis (35). Our data, however, do not indicate whether high bone metabolism rate is the consequence of HAART or whether it is the expression of a direct effect of the HIV infection, because there is evidence for a possible direct role of HIV on bone cells (11). These results might also be the consequence of vitamin D deficiency. However, no alterations of PTH or vitamin D were found in our patients during HAART treatment (our unpublished observation).

In summary, the present data indicate that bone metabolism derangement is present in children on long-term HAART. These alterations might be the cause of the observed low BMD values. Moreover, there was only a partial improvement in bone mass measurements over time. Nevertheless, HIV-infected children and adolescents seem at great risk of not obtaining an optimal bone mass. Therefore, impairments in obtaining an optimal bone mass should be prevented to avoid future complications, and the role of possible therapeutic approach should be urgently assessed.

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