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Altered bone metabolism in children infected with human immunodeficiency virus

G Zamboni[1], F Antoniazzi[1], F Bertoldo[2], S Lauriola[1], L Antozzi[3] and L Tatò[1]

Pediatric Clinic[1], Institute of Internal Medicine[2] and Institute of Infectious Disease[3], University of Verona, Verona, Italy

Zamboni G, Antoniazzi F, Bertoldo F, Lauriola S, Antozzi L, Tatò L. Altered bone metabolism in children infected with human immunodeficiency virus. Acta Pædiatr 2003; 92: 12–16. Stockholm. ISSN 0803-5253

Aim: Data on bone homoeostasis of children infected with human immunodeficiency virus (HIV), at the time of the gain in bone mass, are very rare. To determine possible alterations in bone metabolism, 13 prepubertal vertically HIV-infected children were studied. *Methods:* Viral load, CD4 count, interleukin-6 (IL-6), growth hormone, insulin-like growth factor-I (IGF-I), IGF binding protein-3 (IGFBP-3), acid-labile subunit (ALS), IGFBP-3 proteolysis, osteocalcin in blood and N-terminal telopeptide of type I collagen in urine were determined. Lumbar spine bone mineral density was examined by dual-energy X-ray absorptiometry. *Results:* Low osteocalcin levels were found in all patients. Low IGF-I was found in only six children, who had low CD4 count and high IL-6 levels, with normal levels of IGFBP-3 and ALS, absent IGFBP-3 proteolysis and decreased bone mineral density, irrespective of viral load or growth.

Conclusion: Low serum osteocalcin levels appear to be an initial warning sign of possible altered bone metabolism in HIV-infected children. However, only when the immune system becomes more seriously compromised is bone loss measurable by bone densitometry.

Key words: Bone densitometry, bone metabolism, children, HIV infection, interleukin-6

G Zamboni, Pediatric Clinic, University of Verona, IT-37134 Verona, Italy (Tel. +39 45 8074469, fax. +39 45 8200993, e-mail. Giorgio.Zamboni@univr.it)

Numerous factors may affect bone mineral density (BMD) in childhood (1) and various potential effects on skeletal homoeostasis may result from human immunodeficiency virus (HIV) infection (2). Although the probability of osteoblasts being infected *in vivo* by HIV is very low (3), infection may affect bone metabolism indirectly through decreased physical activity, malnutrition, fat malabsorption and endocrine diseases (4). Moreover, HIV infection is characterized by enhanced levels of proinflammatory cytokines that not only enhance HIV replication and contribute to immunodeficiency, but also play an important role in bone homoeostasis. Some cytokines, such as interleukin-6 (IL-6), may stimulate osteoclast and inhibit osteoblast activity. Therefore, they play a pathogenic role in enhancing bone resorption and inhibiting bone formation (5), and changes in their levels may result in altered bone remodelling. In addition, a decrease in the serum insulin-like growth factor (IGF) system may affect bone remodelling in HIV-infected patients (6). In spite of these potential effects, data on bone metabolism of HIV-infected patients are conflicting, scarce in adults (4, 7–9) and very rare in children (10–12).

On the basis of these observations, the present study was undertaken to identify possible bone alterations in HIV-infected children, because BMD increases during growth and development.

Patients and methods

Thirteen prepubertal children vertically infected with HIV (4M, 9F, mean age 7.8 ± 2.9 y, range 4.08–12.02 y) were enrolled. All were under antiretroviral therapy with nucleoside analogues, but none of them was receiving protease inhibitors. They had no severe concomitant illnesses, nutritional alteration, recent history of extended bed rest, previous diagnosis of metabolic bone or endocrine diseases, liver insufficiency or renal failure, and had not used drugs with known effects on bone metabolism. Disease stage was classified using Centers for Disease Control and Prevention criteria. Informed consent was obtained from one parent or the guardian for each child; assent was also obtained whenever possible. This study was approved by the institutional ethics board.

Auxological parameters [weight, height, growth velocity, body mass index (BMI)] were evaluated, as well as blood HIV RNA level, CD4 cell count, IL-6, growth hormone (GH) secretion [in both the basal condition and after GH-releasing hormone (GH-RH) stimulation], IGF-I, IGF binding protein-3 (IGFBP-3), IGFBP-3 proteolytic activity, acid labile subunit (ALS), osteocalcin and urine levels of N-terminal telopeptide of type I collagen (NTx). Bone age (BA) and lumbar spine BMD were also determined.

Weight was measured to the nearest 0.1 kg using a beam balance, with children wearing light clothing. Height was measured without shoes by a Harpenden stadiometer. Weight, height and growth velocity standard deviation scores (SDS) related to age were calculated from the standards of Sempé et al. (13).

CD4 cell count was determined by flow cytometry. The determination of plasma viral load was performed using an ultrasensitive branched DNA assay (Chiron Diagnostics, Emeryville, CA, USA) with a lower limit of detection of 50 copies ml^{-1}.

Serum IL-6 levels were determined by enzyme-linked immunosorbent assay (ELISA; Endogen, Woburn, MA, USA) according to manufacturer's protocol. The sensitivity of the assay was 10 pg ml^{-1}.

Serum GH was measured using an immunometric chemiluminescence assay (Diagnostic Products Corporation, Los Angeles, CA, USA). The intra- and interassay coefficients of variations (CVs) were $<6.5\%$ and $<6.2\%$, respectively. The sensitivity was 0.0005 pmol l^{-1}. In this laboratory the response to GH stimulation is considered normal when above 0.47 pmol l^{-1}. Serum IGF-I, IGFBP-3 and IGFBP-3 proteolytic activity were determined as described previously (14).

Total serum ALS was measured using an enzyme-linked immunosorbent assay (Diagnostic Systems Laboratories, Webster, TX, USA). The intra-assay CVs for mean serum concentrations of 26.9, 121.7 and 461.4 nmol l^{-1} were 6.1%, 7.5% and 3.8%, respectively. The interassay CVs for mean ALS concentrations of 34.8, 124.8 and 475.6 nmol l^{-1} were 8.6%, 2.8% and 8.9%, respectively. The sensitivity was 1.1 nmol l^{-1}.

Serum osteocalcin was measured by immunometric chemiluminescence assay (Diagnostic Products Corporation). The intra-assay CVs for mean osteocalcin concentrations of 0.45, 2.26 and 14.02 nmol l^{-1} were 2.8%, 2.8% and 4.5%, respectively. The interassay CVs for mean osteocalcin concentrations of 0.45, 2.26 and 14.02 nmol l^{-1} were 3.5%, 3.8% and 7.1%, respectively. The sensitivity was 0.02 nmol l^{-1}.

Urine levels of NTx were measured by an enzyme immunosorbent assay (Osteomark; Ostex, Seattle, WA, USA). Urine specimens were collected between 10.00 and 12.00 h as the second voiding of the day. Assay values were expressed in nanomoles bone collagen equivalents per litre (nmol BCE l^{-1}). The sample results from a single urine collection were normalized for urine dilution by urine creatinine analysis and were reported as nmoles BCE per mmole creatinine. The intra- and interassay CVs were less than 9%. The sensitivity was 20 nmol BCE l^{-1}.

BA was determined blindly by the same observer, according to the method of Greulich and Pyle (15) and expressed in years. BMD at the lumbar spine was measured by dual-energy X-ray absorptiometry (DXA equipped with paediatric software; Lunar Corp., Madi-

son, WI, USA), as reported previously (16). The second, third and fourth lumbar vertebrae were scanned in the anteroposterior projection. Only the third lumbar vertebra was also measured by lateral scan because of possible interference with the assessment of the second and fourth lumbar vertebrae by overlying ribs or iliac crests, respectively. True volumetric BMD (BMDv) was calculated, expressed in grams per cm^3, taking the vertebral body as an ellipsoid cylinder and dividing the bone mineral content obtained by the lateral scan by the body vertebral volume, calculated as: $\pi \times$ width/$2 \times$ depth/$2 \times$ height. Vertebral dimensions (anterior width, depth, and height) were found using software data. The CV for duplicate measurements in normal children at an interval of 1 wk was 1.0% for AP-BMD, 1.8% for L-BMD and 2.9% for BMDv. BMD SDS from the normal population were calculated as: (measured value – mean population value)/SD of the normal population. The BMD SDS above and below the average reference values were expressed as positive and negative BMD SDS, respectively.

Blood IGF-I, IGFBP-3, ALS and osteocalcin levels, urinary NTx levels and the bone densitometric data of the patients were compared with those of a normal prepubertal population of 198 subjects, aged 4–12 y (94M, 104F), with bone age appropriate for chronological age, BMI between 14.5 and 18 kg m^2, normal intake of calcium and phosphate, and normal physical activity.

In normal subjects, IGF-I levels were 18.35 ± 3.8 nmol l^{-1}; IGFBP-3, 85.0 ± 11.9 nmol l^{-1}; ALS, 212.5 ± 82.7 nmol l^{-1}; osteocalcin, 9.44 ± 2.26 nmol l^{-1} in males and 10.08 ± 2.32 nmol l^{-1} in females; and NTx, 363.4 ± 195.5 nmol BCE $mmol^{-1}$ creatinine. BMDv normal values changed from 0.255 ± 0.33 g cm^{-3} in males and 0.267 ± 0.03 g cm^{-3} in females at 4 y of age to 0.293 ± 0.05 in males and 0.313 ± 0.03 g cm^{-3} in females at 12 y of age.

The results are expressed as mean $\pm$ SD. Statistical analysis was performed using unpaired Student's *t*-test with the Bonferroni correction when appropriate.

Results

Table 1 shows the auxological parameters of the patients at the time of entry into the study. All auxological parameters were generally adequate for age and gender.

Table 2 shows immunological and endocrinological parameters and BMDv in the patients. GH levels after GH-RH stimulation showed normal GH secretion. Serum osteocalcin levels in patients (2.89 ± 2.55 nmol l^{-1}) were significantly lower (-2.7 ± 1.6 SDS) than normal values for age and gender ($p < 0.05$). Blood IGF-I, IGFBP-3, ALS levels and urinary NTx levels were higher in patients (IGF-I 25.7 ± 26.1 nmol l^{-1}; IGFBP-3 130.0 ± 42.5 nmol l^{-1}; ALS 321.5 ± 110.0 nmol l^{-1}; NTx 452.9 ± 154.3 nmol BCE $mmol^{-1}$ crea-

Table 1. Auxological parameters in the patients.

Patient	Gender	Age (y)	Weight SDS	Height SDS	BMI (kg m^{-2})	Growth velocity SDS	Bone age (y)
1	F	12.02	1.7	1.1	19.86	0.20	11.0
2	M	6.51	−1.4	−1.6	14.48	−1.10	5.5
3	F	11.89	1.5	1.7	18.73	−0.50	10.5
4	M	7.69	3.1	2.4	18.38	0.65	7.0
5	M	4.44	1.0	0.9	15.72	1.00	3.5
6	F	11.49	3.3	0.5	24.86	−0.80	10.5
7	F	4.08	−1.3	−1.5	15.16	0.20	3.5
8	F	6.09	2.1	1.0	17.16	−1.60	6.0
9	F	8.40	0.2	1.4	14.13	1.00	8.5
10	F	10.54	0.7	−0.4	18.79	−1.50	10.0
11	F	5.88	1.0	−0.1	17.04	0.50	6.0
12	M	9.08	0.6	0.5	16.96	−1.60	9.0
13	F	4.16	1.5	1.2	16.40	−1.50	3.0
Mean ± SD		7.8 ± 2.9	1.1 ± 1.4	0.5 ± 1.2	17.51 ± 2.81	−0.39 ± 1.02	7.2 ± 2.9
Range		4.08 to 12.02	−1.4 to 3.3	−1.6 to 2.4	14.13 to 24.86	−1.6 to 1.0	3 to 11

tinine) than in normal subjects, but the differences were not statistically significant.

Regarding BMDv, 6 out of 13 patients showed negative BMDv SDS values ($−1.0 ± 0.8$); 2 out of the 6 suffered from severe bone pains, and 1 had spontaneous fracture of the left clavicle. In these 6 patients, IGF-I levels ($10.02 ± 3.4$ nmol l^{-1}; $−1.12 ± 0.8$ SDS) and CD4 count ($499.2 ± 87.1 × 10^6$ cells l^{-1}) were significantly lower than in 7 patients with positive BMDv SDS (IGF-I $39.2 ± 18.3$ nmol l^{-1}, $1.85 ± 1.5$ SDS; CD4 $896.7 ± 170.6 × 10^6$ cells l^{-1}; BMDv SDS $1.3 ± 0.6$; $p < 0.05$ for all parameters). IGFBP-3 and ALS levels were also lower in these patients (IGFBP-3 $110.05 ± 22.5$ nmol l^{-1}, $1.33 ± 0.8$ SDS; ALS $285.9 ± 84.5$ nmol l^{-1}, $0.63 ± 0.55$ SDS) than in 7 patients with positive BMDv SDS (IGFBP-3 $147.17 ± 37.2$ nmol l^{-1}, $2.6 ± 0.9$ SDS; ALS $376.3 ± 98.3$ nmol l^{-1}, $1.8 ± 0.9$ SDS), but the differences were not statistically significant. In contrast, in patients with negative BMDv SDS values, IL-6 levels were much higher ($562.5 ± 356.8$ pg ml^{-1}; $p < 0.05$) than in pa-

tients with positive BMDv SDS, in which they were undetectable. In addition, urinary NTx levels were higher in these 6 patients ($510.3 ± 144.8$ vs $403.7 ± 113.5$ nmol BCE mmol^{-1} creatinine), but the difference was not statistically significant. The analysis of IGBP-3 showed no significant proteolytic activity in any patient.

No significant correlation was found in the patients between osteocalcin and IGF-I, IGFBP-3 and ALS, and between osteocalcin and BMD. In contrast, BMDv values were positively correlated with IGF-I levels and CD4 count and negatively with IL-6 levels. Figure 1 shows significant positive correlations among BMDv SDS and IGF-I SDS levels ($r = 0.746$) and CD4 cell count ($r = 0.809$) and a negative correlation between BMDv and IL-6 levels ($r = −0.742$); $p < 0.05$ in all cases.

Discussion

All of the HIV-infected children in this study had

Table 2. Immunological and endocrinological parameters, and volumetric bone mineral density (BMDv) in the patients.

Patient	CDC class	Viral load (copies ml^{-1})	CD4 count (× 10^6 cells l^{-1})	IL-6 (pg ml^{-1})	GH peak (pmol l^{-1})	IGF-I (nmol l^{-1})	OC (nmol l^{-1})	BMDv (g cm^{-3})	BMDv SDS
1	A2	23450	1220	UD	0.53	57.6	0.53	0.361	1.3
2	C3	47639	350	870	0.93	12.7	1.47	0.210	−2.0
3	A1	8931	750	UD	0.68	68.0	6.30	0.354	1.2
4	B2	13448	506	655	1.03	9.2	7.90	0.235	−1.6
5	B1	7630	485	720	1.00	9.2	2.20	0.232	−0.7
6	A1	2794	727	UD	0.47	19.0	0.55	0.350	1.1
7	A1	106377	486	890	0.50	4.1	3.90	0.244	−0.8
8	B2	15450	977	UD	0.64	41.7	1.52	0.331	1.7
9	A2	23450	870	UD	2.28	27.1	1.80	0.298	0.2
10	B1	5067	605	190	0.85	11.5	1.20	0.238	−1.5
11	A1	13918	563	50	0.96	13.4	2.70	0.256	−0.9
12	A2	18918	793	UD	1.04	22.5	6.90	0.345	2.1
13	A2	44580	940	UD	2.09	38.5	0.65	0.308	1.5
Mean ± SD		25512 ± 27920	713.2 ± 245.5		1.00 ± 0.56	25.7 ± 20.1	2.89 ± 2.55	0.289 ± 0.055	0.123 ± 1.42

CDC: Centers for Disease Control; IL-6: interleukin-6; GH: growth hormone; IGF-I: insulin-like growth factor-I; OC: osteocalcin; SDS: standard deviation score; UD: undetectable.
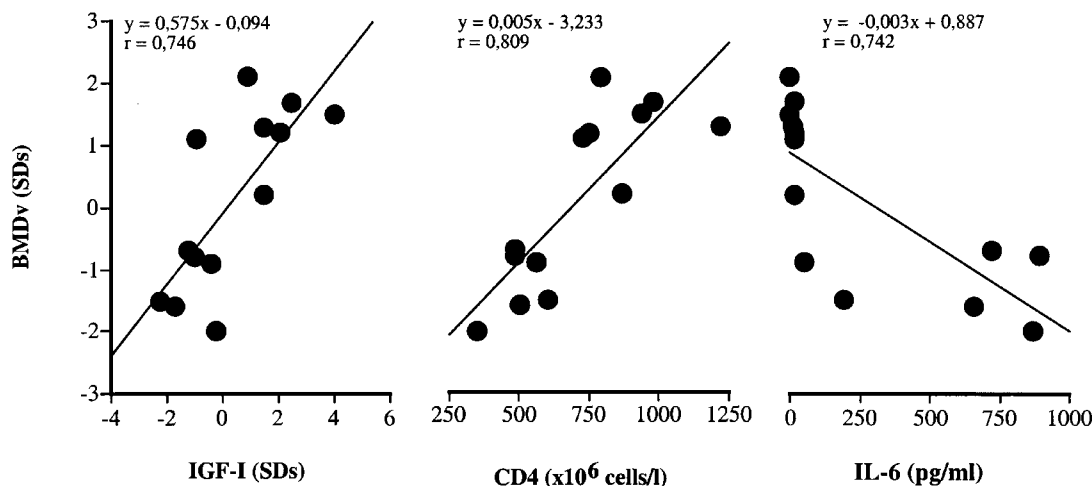
Fig. 1. Correlation between volumetric bone mineral density standard deviation scores (BMDv SDS), insulin-like growth factor-I SDS (IGF-I SDS), CD4 ($\times 10^6$ cells l^{-1}) and interleukin-6 (IL-6, pg ml^{-1}) in the patients.

significantly lower serum osteocalcin levels and slightly higher urinary NTx than controls. Osteocalcin is an indirect parameter of bone formation and NTx of bone resorption. However, a reduced BMD was found in only 6 out of 13 patients, who had a reduced CD4 count, low circulating IGF-I levels despite normal basal and stimulated GH levels, and enhanced IL-6 levels, irrespective of viral load or growth. Alterations in bone markers and bone mineral loss have been reported previously in HIV infection (8–12), but the HIV-infected children (10–12, 17) were studied nearly exclusively in the areas of BMD and of bone metabolism. This study presents new data in the area of GH/IGF-I and immune systems, supporting a relationship between HIV infection and disturbed bone metabolism.

IGF-I is an important regulator of osteoblast and osteoclast activity, modulating osteoblast–osteoclast interactions as a coupling agent in the bone remodelling process. Originally, postnatal longitudinal growth was thought to be controlled by GH and mediated by circulating IGF-I produced by the liver (18); however, recent experiments in mice have shown normal postnatal and peripubertal growth, despite the deletion of the IGF-I gene, the loss of liver IGF-I production and the reduction in circulating IGF-I (19). Recently, importance has been placed upon an autocrine/paracrine role for skeletal IGF-I (18), which is available in the bone matrix and from activated osteoblasts. Nevertheless, the correlation between the increase in skeletal and serum IGF-I levels and the increase in bone mass in children is well known, although in the long bones IGF-I seems to be a major determinant of the cross-sectional area of cortical bone, poorly influencing bone density (20). Low IGF-I levels could be due to a break in the circulating ternary complex (21), comprised of IGF-I, ALS and IGFBP-3, by IGFBP-3 proteolysis. Increased IGFBP-3 proteolysis by specific proteases has been described in HIV-infected adults with advanced disease (22) and in HIV-infected children who failed to thrive (23). However, the patients in the present study had generally normal nutrition and growth, and those with low serum IGF-I showed normal values of ALS and IGFBP-3 and absent IGFBP-3 proteolysis.

In HIV infection, as well as in children's chronic inflammation and infection with normal GH production and decreased IGF-I levels (24), there is an altered expression of cytokines, as well as an overproduction of proinflammatory IL-6 (25), which appear to be relevant to the inflammatory process and in influencing intermediary metabolism, including a negative interference with IGF-I activity or secretion (6, 26). Because circulating IGF-I is produced mainly in the liver and IL-6 is notably involved in the regulation of gene transcription during inflammation, it may be hypothesized that overproduction of IL-6 acts negatively on liver IGF-I gene expression and therefore is responsible for decreased circulating IGF-I, with a possible limited role for the growth factor in bone remodelling. IL-6 hypersecretion, together with low IGF-I levels and low growth velocity (6) or poor growth and reduced energy intake (26), has already been reported in HIV-infected children. In 6 out of 13 patients in the present study, IL-6 levels inversely correlated with IGF-I levels, and CD4 count, did not correlate with viral load or growth impairment.

HIV could reduce bone mineralization by enhancing production of IL-6, an autocrine/paracrine factor which stimulates osteoclast formation and activity, and by reducing the skeletal production of IGF-I. IL-6 overproduction by some viruses has also been implicated in some pathological conditions characterized by bone loss (27). Therefore, increased activity of cytokines, as well as the recently identified osteoprotegerin that promotes osteoclast activation (28), may also play an

important role in mediating disturbed bone metabolism during HIV infection.

In conclusion, a low serum osteocalcin level appears to be an initial warning sign of possible altered bone metabolism. Only when the immune system is more seriously compromised, as indicated by high IL-6 levels and a more severe decrease in CD4 count, does bone loss become measurable by bone densitometry. HIV infection itself, by enhancing cytokine production, appears to be responsible for altered bone metabolism.

These results need to be confirmed in a larger cohort of patients, both to demonstrate conclusively that HIV infection could result in a possible bone disorder at a very critical period of life with respect to reaching maximal peak bone mass, and to develop treatment strategies to preserve maximal bone mass in patients at risk for reduced BMD.

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