

# HIV infection is associated with low bone quality, independently of bone mineral density

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## Summary:

Microindentation is a new technique to assess bone quality. We report significantly worse bone quality, as measured by microindentation, in treatment-naïve HIV patients than in healthy controls. This deterioration in bone health was not detectable by measuring BMD.

## Conflicts of Interest and Source of Funding

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## **Abstract**

### **Introduction**

Low bone mineral density (BMD) in HIV-infected individuals has been documented in an increasing number of studies. However it is not clear whether it is the infection itself or the treatment that causes bone impairment. While BMD is a widely used surrogate for bone fragility, other techniques can provide a more accurate measure of bone quality. One of these techniques is microindentation, which measures bone material strength (BMSi) directly.

### **Methods**

To test the hypothesis that HIV-infected patients have poorer bone tissue mechanical properties (bone tissue quality) than controls, we carried out a bone microindentation study in a cohort of HIV-positive patients, and a sample of age- and sex-matched controls.

### **Results**

We recruited 85 patients, 50 infected with HIV and 35 controls. HIV-infected patients had significantly lower BMSi (84.9, SD 5.7) than controls (90.2, SD 6.2;  $p < 0.001$ ), and thus worse bone quality at the tissue level. In contrast, we observed no significant difference in BMD between cases and controls at any of the sites examined (total hip, femoral neck, and lumbar spine).

### **Conclusions**

For the first time, we describe *in vivo* measurement of bone quality in HIV-infected patients. We found that HIV infection is associated with bone damage, independently of ART and BMD.

## Introduction

Low bone mineral density (BMD) in HIV-infected individuals has been documented in an increasing number of studies(1–3). The mechanisms behind this association are not clear: both the HIV infection itself and/or the toxicity of Antiretroviral Therapy (ART) toxicity have been implicated, but without conclusive evidence. Population-based studies have shown an association between HIV infection and increased fracture risk (4–6), although most of these studies did not evaluate the role of ART, and no conclusions could be drawn about which factors are ultimately responsible for the increased fracture risk. Clinical studies with ART show weak association between fracture risk and treatments like tenofovir and lopinavir/r (7), but even in those cases, fracture risk could not be explained by low BMD alone. In fact, there is no convincing evidence on the true underlying mechanisms of bone disease in these patients, or even whether this is a real association, or due simply to confounding by other risk factors for osteoporosis and fracture, such as illicit drug use, corticosteroids, alcohol, or low body mass index (BMI).

In clinical practice, measurement of BMD using dual-energy X-ray absorptiometry (DXA) is the most widely used technique for diagnosing osteoporosis. BMD and fracture risk have a close inverse correlation in the general population, particularly in untreated individuals (8). However, while ~50% of women with non-vertebral fractures have a BMD T-score that exceeds the diagnostic threshold for osteoporosis ( $-2.5$ ), less than half of patients that meet the criteria for osteoporosis will suffer a fracture (9). Therefore, DXA provides limited information on bone health, and no information about other key components of bone quality, such as bone strength, composition or microarchitecture.

Recently developed techniques for assessing bone microarchitecture (10,11) provide a better understanding of bone structure, but do not allow assessment of bone material properties in the clinic because samples are taken invasively and have to be tested *ex vivo*. Microindentation is a technique that quantifies a patient's bone material properties at the tissue level, and can be performed in the clinical setting. This technique discriminates between patients with and without fractures (4,12), and has been shown to detect bone tissue alterations in other situations where BMD is relatively preserved despite increased fracture risk, such as fragility fractures in patients with osteopenia (13), Diabetes Mellitus (14), or who are undergoing glucocorticoid treatment (15).

Since the underlying mechanisms of the bone abnormalities observed in HIV-infected patients are not yet well characterized, we carried out a bone microindentation study in a cohort of HIV-positive patients, and a sample of age- and sex-matched controls. We hypothesised that HIV-infected patients have poorer bone tissue mechanical properties (bone tissue quality), than controls. Thus, the primary objective of the study was to test for differences between HIV cases and controls in the Bone Material Strength index (BMSi), as measured by microindentation. Our secondary objectives were to test for differences in Bone Mineral Density (BMD), markers of inflammation, and bone turnover between these groups, as well as the general safety of microindentation in this series.

## Patients and Methods

### *Patients*

We conducted a cross-sectional study of outpatients of the Infectious Diseases Department at our institution, Hospital del Mar, Barcelona, Spain, and healthy volunteers. The study was approved by the local Clinical Research Ethics Committee, and all participants gave written informed consent (Ethical Committee number 2013/5250/I).

Patients were offered the opportunity to participate in the study if they were HIV-positive. All HIV-infected patients were viremic (PCR for HIV >40 copies/ml) and had no prior history of ART (all were treatment naïve). Individuals were excluded from the study if they had a history of treatment with bone-active drugs, or if they had any disease or condition, apart from HIV, that could interfere with bone metabolism, such as structural liver disease, alcoholism, malignancy, Cushing syndrome, hypogonadism, hyperthyroidism, glucocorticoids therapy, hypopituitarism, hyperparathyroidism, chronic kidney disease (any stage), severe chronic obstructive pulmonary disease, co-infection with chronic hepatitis C or chronic hepatitis B, Type 1 diabetes, severe neuropathic disease, and use of opioids or intravenous drugs.

During the inclusion visit, we obtained data on prior fracture, smoking, alcohol consumption, and anthropometry, and took a full medical and medication history. We obtained blood samples from both cases and controls, and made serum determinations of total 25-hydroxyvitamin D, calcium, phosphorus, creatinine, thyroid stimulating hormone, aspartate transferase, alkaline phosphatase, bone turnover markers (bone specific alkaline phosphatase, amino terminal propeptide of type I procollagen (PINP), C-telopeptide), parathyroid hormone, and proinflammatory markers (high sensibility C-reactive Protein, erythrocyte sedimentation rate (ESR), lactate dehydrogenase (LDH), beta-2 microglobulin). Bone Mineral Density (BMD) was measured by DXA (DXA Hologic QDR4500SL™ (S/N 45329)) on the same day as the blood sampling and microindentation test. In HIV patients we also determined CD4+ count, HIV viral load by PCR, time of diagnosis and opportunistic infections. We performed lateral X-Ray of the thoracic and lumbar spine in all participants (controls and HIV patients), to rule out previous fractures.

### *Bone Microindentation Testing*

Microindentation testing was performed using a handheld OsteoProbe™ Reference Point Indenter (Active Life Scientific Inc., Santa Barbara, CA, USA), which consists of a head unit with a displacement transducer, and an impact mechanism. After applying local anesthesia (2% mepivacaine), a sterilized stainless steel probe with a 90 degree conical tip (375µm diameter; <4µm tip sharpness radius) is placed on the midpoint of the midshaft anterior tibial plateau, and inserted through the soft tissue until it makes contact with the bone surface. Holding the device perpendicular to the bone surface (fig 1. A), the operator displaces the device until, after a preload force of 10N, the device's trigger mechanism releases a 30N impact force. This displaces the test probe into bone, and the displacement transducer measures the indentation distance (ID). After 8 repeated indentations separated by

approximately 2 mm, the operator performs 5 additional indentations with the same probe on a cube of polymethylmethacrylate (PMMA) (fig 1.B). The software provides the BMSi, defined as 100 times the ratio between the harmonic mean ID of the five impacts into the calibration phantom (PMMA) and that of the 8 impacts into the bone. This technique has previously been validated in humans (12-15). The procedure takes less than five minutes, causes minimal discomfort to the patient, and no complications have been observed in published studies (12-15).

#### *Laboratory tests*

Bone turnover markers: Amino pro-peptide of type 1 collagen (P1NP) (mg/L) was measured by RIA (interassay CV, <10%; Immulite 2000 (Siemens)), C-telopeptide of type I collagen (CTX; ng/mL) measured by ELISA (interassay CV <8%; Roche Diagnostics, Indianapolis, IN, USA), bone specific alkaline phosphatase measured by ELISA (BSAP; interassay CV <10%; Roche Diagnostics, Indianapolis, IN, USA), intact parathyroid hormone as measured by chemiluminescent immunoassay (CLIA) technology (iPTH, interassay CV <10%; Immulite 2000 (Siemens)), 25-OH Vitamin D measured by CLIA (interassay CV <10%; Elecsys Vitamin D Total Assay (Roche Diagnostics, Indianapolis, IN, USA)) and also were measured proinflammatory markers: high sensitivity C-reactive Protein measured by CLIA (hs-CRP; Immulite 2000 (Siemens)), erythrocyte sedimentation rate (ESR), beta-2 microglobulin measured by CLIA (Immulite 2000 (Siemens)) and procoagulation markers D-dimer measured by immunoturbidimetric assay (HemosIL D-Dimer, Instrumentation Laboratory, (ACL TOP300)) and fibrinogen (Clauss method Fibrinogen-C, Instrumentation Laboratory, (ACL TOP300)).

#### *Statistical analysis*

We compared quantitative and categorical variables between HIV-infected and control groups using two-sample t-tests, and  $\chi^2$ -test, respectively. The nonparametric Fisher–Pitman test for paired independent samples were used in the case of the analysis of a subset of patients in the HIV group due to the reduced sample. We performed univariate and multivariate linear regression to test for association between BMSi and HIV status. Factors with a univariate *P*-value of <0.20 were included in the multivariable model. We explored interactions in the HIV group between baseline BMSi and sex, age, nadir CD4 count, and time since diagnosis. Assuming a type I and II error of 0.05 and 0.2 in a two-sided test, respectively, 15 controls and 22 HIV patients were required to detect a statistically significant difference of  $\geq 5$  units of BMSi. The common standard deviation was assumed to be 5, and we expected a drop-out rate of 10%. Results with  $p < 0.05$  (two-tailed) were considered to be statistically significant. Analyses were carried out using Stata/IC 13.1.

## Results

We recruited a total of 85 study subjects (50 HIV-infected patients, and 35 controls) between January and October 2014. The time since diagnosis of infection ranged from 0 to 19 years (mean age at diagnosis, 34 years), and there was no significant difference between cases and controls in the sex ratio, mean age, or mean. We observed no remarkable difference in smoking and alcohol habits between cases and controls, and no prior or current use of intravenous drugs. Baseline characteristics of the sample are presented in Table 1.

We observed no differences in bone turnover markers, either of bone formation (P1NP, bone specific alkaline phosphatase) or resorption (C-Telopeptide; Table 2). HIV patients had significantly lower levels of 25-hydroxy-vitamin D than controls (22.6 vs 30.2,  $p<0.015$ ).

In contrast, levels of the proinflammatory markers high sensibility C-Reactive Protein (0.48 vs. 0.12;  $p=0.005$ ) and ESR (24.1 vs 5.2  $p=0.0001$ ) were significantly higher in HIV-infected individuals than in controls; similarly for other procoagulation markers such as D-dimer (289.6 vs 137.2,  $p=0.018$ ), fibrinogen (386.6 vs 340.3;  $p=0.037$ ) and beta2 microglobulin (2.59 vs 1.438,  $p=0.0001$ ), respectively.

Compared to controls, HIV patients had significantly lower BMSi, and thus worse bone quality at the tissue level. BMSi was 84.9 (5.7) in HIV-infected patients and 90.2 (6.5) in controls ( $p<0.001$ ; Fig 1).

The associations observed between HIV and BMSi in the univariate analyses were maintained in multivariable analysis (Table 3). In contrast, we observed no significant differences in BMD between cases and controls at any of the sites examined (total hip, femoral neck, and lumbar spine; Table 2).

Four of the HIV patients (8%) had existing lumbar spine fractures, and significantly lower mean (SD) BMSi, [77.5 (2.2)] than the non-fracture group [85.36 (5.3);  $p=0.021$ ]; however, we observed no significant difference in lumbar spine BMD between these subgroups [non-fracture, 0.989(0.12); fracture 0.933 (0.07);  $p=0.653$ ].

We tested for pre-specified interaction between HIV and microindentation with age, sex, nadir CD4 count, and time since diagnosis, but found no interactions.

There were no complications in any patient during or after the microindentation procedure.

## Discussion

In this paper we observe poorer bone quality in HIV-infected patients than in controls, as measured by *in vivo* microindentation; this effect is independent of BMD and ART. Moreover, HIV-infected patients present higher levels of markers of inflammation, and this persistent inflammatory status has previously been found to be associated with bone fragility in other conditions (16,17).

Bone disease associated with HIV infection is an increasingly recognized clinical problem, with reports of increased prevalence of osteopenia, osteoporosis, increased risk of fragility fractures, and lower BMD (5,6,18,19).

However, we found that bone density (by DXA) in our series of treatment-naïve HIV-patients was similar to that in age-matched, uninfected controls after adjustment for confounding factors (BMI, smoking). The main difference between ours and previous studies is that we did not observe differences between these groups in their profile of traditional risk factors (i.e. alcohol consumption, corticosteroid treatment, BMI, tobacco use, intravenous drug use, and malnutrition). This absence of differences in may be because the profile of HIV patients has changed over time, in that HIV is now mainly transmitted through sexual activity, whereas in the past the transmission mechanism was mainly druguse(2,18).

Population-based studies show that the prevalence of fractures among HIV patients is significantly higher than in individuals without HIV (6,18,19).However, these studies did not consider individual clinical information, BMD, or many relevant HIV-specific clinical parameters, and did not differentiate between traumatic and fragility fractures. Thus, these authors only observed a general association between HIV and lower bone quality, but were unable to establish the underlying causalmechanism.

Bone loss has been found to be especially notable after beginning ART. We found that HIV patients already show abnormal BMSi during the early pre-treatment stages of disease, even though their BMD is not significantly different to that in controls, as observed inprevious studies. Grijzen *et al.* found that HIV infection was not associated with BMD, which suggests that bone loss in HIV may begin before the HIV infection itself, due to prior exposure to risk factors (20).

In contrast, other studies have reported low BMD in HIV patients, with a meta-analysis showing that HIV-infected patients have a 6.4-fold higher odds of having low BMD (>1SD below the mean) than controls (21). Since most previous studies have used healthy controls with significant differences in life-style and in exposure to classical risk factors for low BMD, it is probably not informative to compare these populations, even after adjustment. Indeed, several classical risk factors for low BMD are more prevalent among HIV patients, such as smoking, alcohol consumption, low BMI, and drug abuse (21,22). In our study,the HIV cases had a similar risk factor profile to controls, and consequently there was no significant

difference in BMD between these groups. Thus, BMD seems to be reduced in the presence of these traditional risk factors, and HIV infection is simply another factor to be added.

However, while BMD is considered the gold standard for evaluating bone health, in specific clinical situations, impaired quality of bone material is the most prominent feature rather than the amount of mineral (13–15), and bone fragility is only partially assessed by densitometry (9). In addition to finding no differences in BMD in our study, we also found no differences in bone turnover markers, a similar result to that of Grijzen *et al.*, who reported high prevalence of low BMD in HIV-infected men in the absence of markers of increased bone turnover (20).

Until recently, direct mechanical testing of bone strength has been cumbersome, requiring an invasive procedure to extract bone samples for *ex vivo* laboratory testing. Recently, microindentation, a minimally invasive technique based on microscopic mechanical challenge on the bone surface, has permitted direct measurement of the tissue's mechanical performance (i.e., strength vs. fragility). This technique induces separation of mineralized collagen fibrils and initiates microcracks, which are likely to be the mechanism of fracture initiation (12). Hence, this technique measures the mechanical competence of bone tissue to resist the initiation and propagation of fractures (4,12,13). As we expected, HIV-infected patients show lower BMSi values, independently of BMD. This indicates that the material properties and bone quality are diminished, even though the amount of mineral, as measured by BMD, remains at a similar level. The absence of differences in classical risk factors (alcohol, smoking, malnutrition, drugs abuse, etc.) between cases and controls further supports the idea that this deterioration is due to the HIV infection.

We observed a moderate association between bone metabolism and the immune system, which could ultimately explain our findings. Osteoclasts, the cells responsible for bone resorption, share a common origin with monocyte pre-cursors, and respond to the same regulators (23), such as receptor activator of NF- $\kappa$ B (RANK).

HIV infection results in extensive damage to the immune system, with continuous immune activation leading to chronic inflammation and increased activation of mature B cells (24). This in turn results in increased RANKL levels (24), more rapid bone destruction, and a diminished population of resting memory B cells, which are responsible for the synthesis of OPG, an inhibitor of RANK.

Studies in untreated HIV patients have assessed whether bone is affected through direct viral effects as well as inflammatory effects. Fakruddin *et al.* found that HIV virus proteins like gp120 or Vpr directly stimulate osteoclast activity (25,26), and Cotter *et al.* found that p55-gag induces osteoblast apoptosis (27). Moreover, inflammatory cytokines such Tumor Necrosis Factor  $\alpha$  or Interleukin 6 promote osteoclast maturation and, thus, bone resorption (28). This complex regulatory signalling network at the immuno-skeletal interface, with the potential impact of inflammatory changes, may alter bone mechanical properties by affecting bone tissue quality, bone turnover, and ultimately bone fragility (29). Using bone



microindentation, we can detect early changes in bone properties well before current clinical measurement instruments can, possibly because bone is affected from the very beginning of the infection due to its close interaction with the immune system. A similar effect has been observed in other diseases such as Type 2 diabetes (14), and in response to glucocorticoid treatment (15), where bone quality changes are measurable.

Our results represent a new line of research into bone disease in the context of HIV infection. Since we can quantify a distinct dimension of bone strength, the mechanisms that underlie this phenomenon can be explored in depth and, more importantly, treatments or strategies to prevent bone loss and fractures can be developed. The long-term survival of HIV-infected individuals increases their exposure to complications such as fragility fractures. Similarly, further study of bone quality after initiating ART are also required.

This study has some limitations that must be taken into account. First, this is a single centre study, with a relatively limited number of patients. Second, while this technique is well validated and is receiving increasing interest in many areas, it is not yet possible to make clinical decisions based on its findings.

This study also has various strengths, such as the fact that we have been able to better isolate the impact of the infection itself, without the potential interference of ART or confounding due to lifestyle factors or concomitant diseases. The use of the microindentation technique is also an important advantage, in that it allows us to measure bone quality directly, and to capture a new aspect of bone strength.

## **Conclusions**

In summary, for the first time, we describe in vivo measurement of bone quality in HIV-infected patients. Microindentation captures changes in bone quality that are not measured by BMD. We found that HIV infection induces bone damage itself, independently of ART and BMD.

### **Funding**

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## Figure Legends

Figure.1. Microindentation testing performed using OsteoProbe™ (Active Life Scientific Inc., Santa Barbara, CA, USA). A.Placed on the midpoint of the midshaft anterior tibial plateau, and inserted through the soft tissue until it makes contact with the bone surface. B.After the measurement in the bone the operator performs 5 additional indentations with the same probe on a cube of polymethylmethacrylate (PMMA).

Figure 2. Box-plot with BMSi comparing controls and HIV +. Boxes indicate the interquartile range and the median. The bars are the range (lowest and highest values). Outliers values are represented as points.

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