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FEM CONSTAR,

Que la Tesi Doctoral titulada: "*In vivo* microindentation for the assessment of bone material properties", presentada pel llicenciat **Robert Güerri Fernández** i dirigida per nosaltres, representa una aportació rellevant al tema i reuneix mèrits suficients per a ser presentada i defensada davant del Tribunal corresponent.

I perquè així consti, signem la present, a Barcelona, dos d'abril de dos mil tretze

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In vivo microindentation for the assessment of bone material properties

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Abbreviations

AFF-Atypical Femoral Fractures

AUC-Area Under the Curve

BMD-Bone Mineral Density

BP-Bisphosphonates

CI-Confidence Interval

CreepID-Creep Indentation Distance

DEXA-Dual X-Ray Absortiometry

du-Ultimate Displacement

E-Young's Modulus

FN-Femoral Neck

Fu-Ultimate Force

IDI-Indentation Distance Increase

LS-Lumbar Spine

NIH-National Institutes of Health

OI-Osteogenesis Imperfecta

S-Structure

TotalID-Total Indentation Distance

U-Area Under the Load-displacement Curve

WHO-World Health Organization

1. BACKGROUND

1.1 Epidemiology of Osteoporosis

Osteoporosis has been defined as a systemic skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (1)

The first vestiges of osteoporosis in Europe were reported in a bone of the Pleistocene (700,000 BC), from the jaw of a Neanderthal man, the *Homo Heidelbergensis* (2). In remains from the Bronze Age found in Franzhausen, Lower Austria, the skeleton of a middle-aged woman had decreased bone mineral density (BMD) in the hip. In 1680, Crisóstomo Martínez Sorlí (1638-1694) authored an anatomical atlas that depicted the deterioration of bone structure with age. In the 19th century, Sir Astley Paston Cooper described the association between bone deterioration and hip fracture in 1822; A few years later, in 1829, the surgeon and pathologist Jean Georges Chrétien Frédéric Martin Lobstein coined the term *osteoporosis*.

Since then, there has been an increasing awareness about this impairment of bone and its worst consequence, fractures. Nowadays, osteoporosis is a major public health problem through its association with fracture.

Clinically, osteoporosis is recognized by the occurrence of characteristic low trauma fractures. Therefore, any meaningful definition of osteoporosis must take into account this risk of fractures after a minor trauma, the so-called fragility fractures. In fact, fragility fractures at any location (wrist, ribs, vertebrae, and hip) are a devastating problem, associated with considerable morbidity, a decline in quality of life, and increased mortality (3). In the United States, approximately 1.5

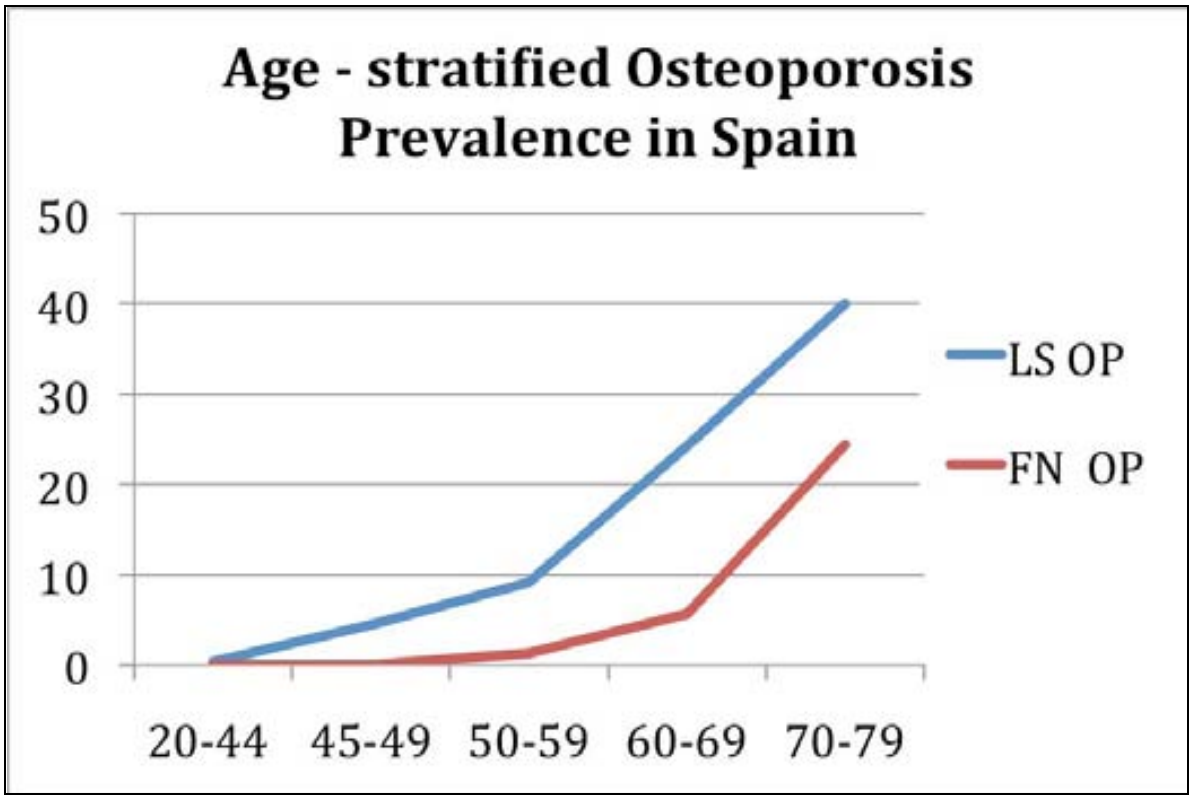
million fractures annually are attributable to osteoporosis(4), whereas in the European Union, in the year 2000 the number of osteoporotic fractures was estimated at 3.79 million, of which 0.89 million were hip fractures (5).

The female-to-male ratio of hip fractures is approximately 2:1 (6, 7) and the occurrence increases exponentially with age. However, the incidence of wrist fracture ranges from 400 to 800 per 100,000 women but remains stable through the last decades of life(7). Vertebral fractures are much more difficult to estimate because these are often asymptomatic.

The overall prevalence of osteroporosis is increasing. It is estimated that around 40% of all U.S. white women and 13% of U.S. white men aged 50 years will experience at least one clinically apparent fragility fracture in their lifetime(8). These estimates predict that 35% of women will have a vertebral fracture, 18% a hip fracture, and 17% a Colles fracture. Hip fracture will be recurrent in 14% of women and 25% will have multiple vertebral fractures (9). The lifetime risk of fracture in women over 50 may be as high as 70%.

Looking at the Spanish population, Díaz Curiel et al. studied the prevalence of osteoporosis stratified by age (figure 1). They report increased prevalence of osteoporosis with age: 11.13% (95% confidence interval (CI) 9.4-12.8%) at lumbar spine (LS) and overall prevalence of 4.29% (95% CI 3.2-5.4%) at femoral neck (FN). They estimate that 12.73% of the Spanish female population had osteoporosis at LS or FN, which represented 1,974,400 women in 2000 (10).

Fig 1. Prevalence of osteoporosis stratified by age in the Spanish population.



1.1.1 Hip Fracture

Hip fracture is the most serious consequence of osteoporosis, and the associated costs are high. Hip fracture induces more disability than any other type of osteoporotic fracture, and incidence increases exponentially with age in both sexes. In women, rates increase from 2/100,000 person-years before 35 years of age to 3,032/100,000 person-years at 85 years and older; in men the rates are 4 and 1,909/100,000 person-years, respectively (11). Meanwhile, in Spain, the average incidence of hip fracture in 2004 was 6.94±0.44 hip fractures per 1,000 inhabitants/year (95% CI, 6.07–7.82). Adjusted for sex, the incidence was 4.17±0.26 per 1,000 inhabitant-years in men and 9.13±0.66 per 1,000 inhabitant-years in women (12).

1.1.2 Vertebral Fracture

The epidemiology of vertebral fracture is less well characterized than that of hip fracture, predominantly due to the lack of universally accepted diagnostic criteria. In addition, substantial proportions of vertebral fractures are asymptomatic and therefore escape clinical detection.

The incidence of vertebral fracture increases with age in both sexes. Most studies indicate that the prevalence of vertebral fracture in men is similar to, or even greater than, that seen in 50- or 60-year-old women (13). The age-adjusted prevalence of radiological fracture has been estimated at 8±25% in women over 50 years of age, depending on the definition used (13).

In a cross-sectional study in Spain, the estimated prevalence among women was 21.4% (95% CI: 17.7%-25.1%) for all vertebral fractures and 9.7% (95% CI: 6.7%-12.7%) for moderate-severe fractures. In women over the age of 75, the respective values were 46.3% (95% CI: 34.2%-58.3%) and 23.9% (95% CI: 13.6%-34.2%)(14).

1.1.3 Distal Forearm Fracture

Wrist fractures are the most common fractures sustained by postmenopausal women (15) and the age-adjusted female-to-male ratio is 4:1, with 85% of these fractures occurring in women (16). Incidence of wrist fractures increases rapidly after menopause in women, reaches a plateau at around 65 years of age, and then remains stable for the next decades of aging.

Taking into account all these data, there is an increasing concern about the economic cost of osteoporotic fragility fractures. These fractures impose a considerable financial burden on health services due to reduced mobility, hospitalization and rehabilitation (17). The cost of osteopososis is considered to

have three components: 1. Direct costs of the surgical treatment plus the initial rehabilitation due to fracture, called “first-year cost”; 2. Indirect costs of the fractures, i.e., the cost of fractures sustained before a specific year but still inducing costs in that year, called “long-term disability cost”; and 3. The cost of pharmacological fracture prevention including administration and monitoring costs, the “pharmacological fracture prevention costs”.

Strom et al studied the incidence and costs of osteoporosis across Europe in 2010. In Spain it was estimated that approximately 204,000 new fragility fractures were sustained, comprising 40,000 hip fractures, 30,000 vertebral fractures, 30,000 forearm fractures, and 104,000 other fractures. The economic burden of incident and previous fragility fractures was estimated at €2.842 million for the same year. Incident fractures represented 48% of this cost, long-term fracture care 37% and pharmacological prevention 15%(18).

Previous and incident fractures also accounted for 70,800 quality-adjusted life years (QALYs) lost during 2010. Prior fractures accounted for 57% of the total loss and 60% of the loss occurred in women. The monetary value of a QALY varied from 1 to 3 times the gross domestic product (GDP) per capita. Assuming a QALY is valued at 2 times GDP/capita, the total cost of the QALYs lost was estimated at € 3.27 bn.

1.2 The Diagnosis of Osteoporosis

With the implementation of BMD measurement by dual energy X-ray absorptiometry (DEXA) the World Health Organization (WHO) defined osteoporosis based on BMD levels. Taking as a reference the values of a young adult population, T-score values were defined, which represents the absolute standard deviation with respect to the mean BMD of a young adult. Osteoporosis

is defined as a T-score at or below -2.5 (1,2,3,4). In other words, the threshold level of BMD for osteoporosis is 2.5 standard deviations below the average BMD of a young adult. The DEXA measured at the hip or LS is the most common BMD measurement.(17)

Many epidemiological studies have indicated that fracture risk increases with decreasing BMD in untreated individuals. In an analysis involving 90,000 person-years of observation and more than 2,000 fractures, a drop in BMD of 1SD below the age-adjusted mean predicted a relative risk of 1.5 or more for fracture(19). Hence, BMD in untreated individuals could be a useful surrogate marker for bone strength and for prediction of fracture risk (19). However, BMD does not identify all individuals at risk. For instance, about half of elderly women who present with nonvertebral fractures would not be classified as 'osteoporotic' because they have hip and spine BMD above the –2.5DE T-score threshold, measured by DEXA (20). On the other side, the proportion of fragility fractures attributable to low BMD (indicating reduced bone strength) remains modest (from 0% to 44%) (21, 22). Consequently, this lack of correlation between BMD and fractures opens the possibility that other factors in addition to density could affect bone propensity to fracture.

In fact, BMD is only a modest risk factor for fractures. Some authors assert that up to 85% of fracture risk in general, or to the rise in fracture risk with age, is unrelated to BMD (21, 23). That is why the concept of bone quality has been strengthened by the identification of a number of risk factors for fracture that are independent of BMD. Clinical risk factors that contribute to fracture risk independently of BMD include, among others, age, previous fragility fracture,

premature menopause, a family history of hip fracture, and the use of oral corticosteroids (24).

Besides BMD, other factors contribute to bone strength. In 2001, the National Institutes of Health (3) Consensus Panel defined osteoporosis as “a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture”. Bone strength, in turn, was stated to be influenced by both BMD and bone quality.

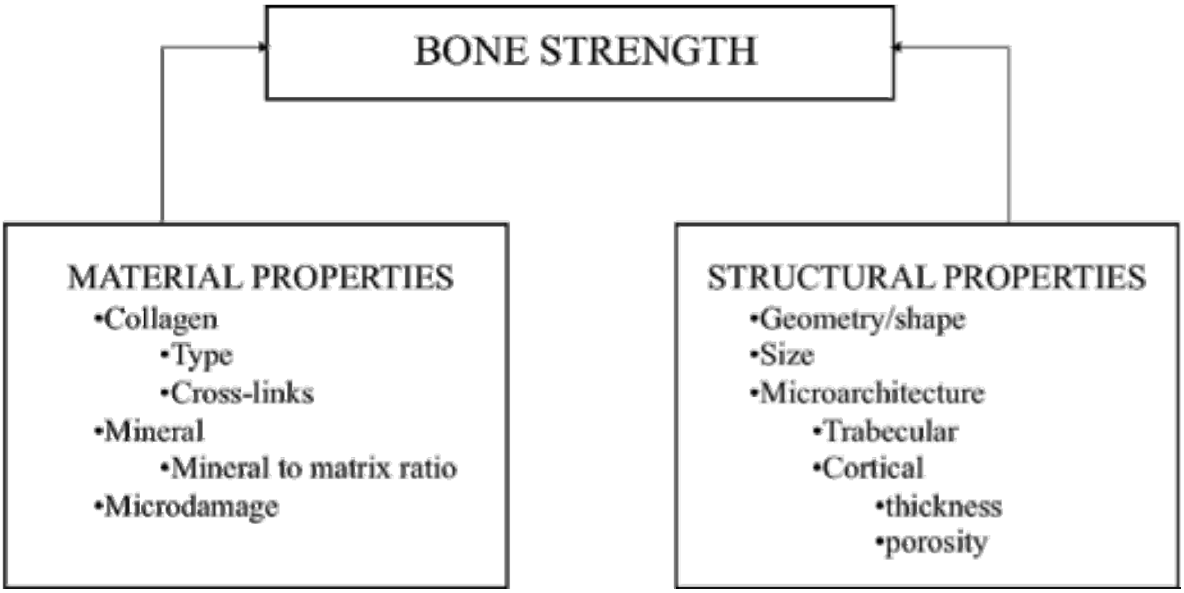
1.3 Bone Quality

Bone quality is not precisely defined. It could be described as a collection of all the factors that determine the behaviour of skeleton in response to stress and its propensity to fracture. The concept implies microarchitecture, accumulated microscopic damage, quality of collagen or size of mineral crystals (fig 2). A common denominator of most of these elements is the rate of bone turnover. Alltogether, bone quality encompasses a set of characteristics that influence bone strength apart from BMD.

There are structural and material properties that determine bone strength (fig 2). The structural properties that affect bone quality include geometry and size of the bone, trabecular architecture, cortical thickness and cortical porosity (25). On the other side, the material properties of bone are the characteristics of the collagen matrix in which collagen is embedded and also the characteristicis of the mineral crystal itself. Nevertheless, what has more importance is how these two properties are combined. Moreover, the appearance of microscopic cracks in the collagen and mineral strcuture can also affect the biomechanical properties of the bone tissue. The rate of bone turnover is extremely important in this process. The

constant remodeling is responsible for the repair of microcracks and replace old bone tissue (26), and also influences bone strength.

Fig.2. The influence of structural and material properties in the quality of bone (Adapted from Felsenberg et al.)



1.3.1 Structural Properties

1.3.1.1 Geometry

The distribution of bone mass throughout the bone influences bone geometry and shape. This distribution of mass affects mechanical behaviour, i.e. the greater the inner cross-sectional diameter of the bone, the more this bone will be able to resist bending and torsion loads.

One remarkable property of bone is the ability to adapt its geometry to the direction of the normal physiological forces (Wolf's law).

1.3.1.2 Microarchitecture

Bone is composed of trabecular and cortical bone. Different bones across the anatomy have different proportions of these two types of bone. Vertebral bodies contain a high proportion of trabecular bone, which has a 3-D network-like structure composed of trabeculae organized as rods or plates (26). The aim of this trabecular bone is to distribute the applied forces. Bone distributes the amount of material in an anisotropic structure adapted to the direction and amount of force, optimizing the structure and gaining the optimal strength with a minimum of material. The trabecular network can develop millions of structures with similar strength, giving to the whole structure a high resistance.

On the other side, cortical macro and microarchitecture is mainly characterized by the diameter of the bone, thickness, cross sectional area of the cortex, and the number and diameter of Haversian canals (27). The cortical porosity, due to a

high number of large Haversian canals within the cortical bone, makes the structure less strong and more likely to fracture (28).

1.3.2 Material Properties

Bone tissue is a two-phase porous composite material primarily constituted of collagen and mineral. The mechanical properties are determined by the amounts, arrangement, and molecular structure of these primary constituents. The mineral component confers strength and stiffness to the tissue. The collagen component is tough and improves bone's work to failure or toughness. The ratio of mineral to collagen in bone affects both bone's strength and brittleness. Excessive mineral content, or a change in quality of the mineral, increases brittleness and is detrimental for bone material properties. Collagen has a small influence on the strength and stiffness of bone, but mostly improves bone's toughness (29). The most obvious clinical example of the mechanical effects of a collagen defect is osteogenesis imperfecta (OI).

Another constituent of bone strength is a constant induction of microdamage in the bone material tissue. Normal sub-maximal loading on bone causes microdamage that appears in the form of microcracks, which are a means of absorbing energy from the load without causing complete fracture of the bone(30, 31). Microcracks accumulate with age in both trabecular and cortical bone, which may be related to the increased fracture risk (32) . The presence of microcracks stimulates targeted remodeling (32) and under normal conditions microcracks are repaired as fast as they are produced (33). When microcracks form faster than they are repaired bone mechanical properties may be adversely affected. The rate at which microcracks are repaired influences bone strength (30).

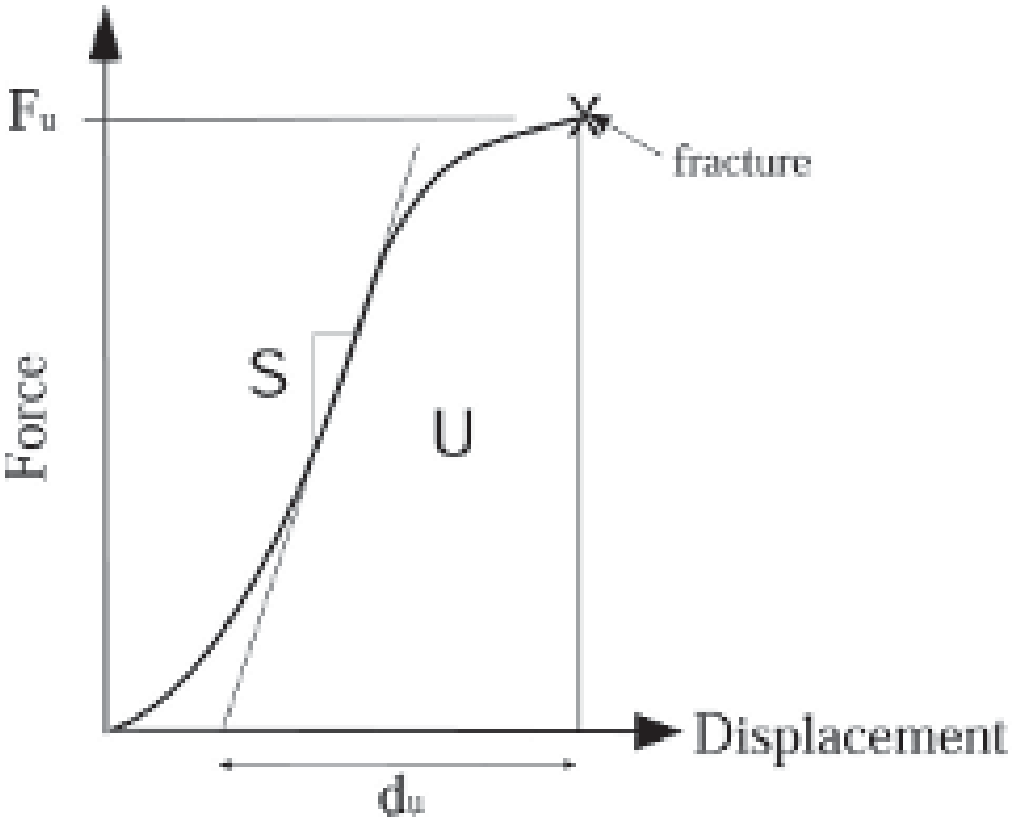
1.3.3 Measurement of Material Properties

From a mechanical perspective, fractures represent a structural failure of the bone, where the forces applied on it exceed its load-bearing capacity. The ability of a bone to resist fracture depends on the aforementioned determinants of bone quality, the amount and structural properties, including the spatial distribution, of bone mass and the intrisic material properties of bone tissue. It must be considered both: material and structural properties in the the evaluation of the effect of a disease or a therapy on bone strength. Therefore, assessing the bone material properties may be critical for understanding mechanisms that underlie changes in whole-bone properties.

For that purpose, a number of biomechanical parameters are available to characterize the integrity of bone. The main biomechanical properties describe the relationship between load applied to a structure and displacement in response to the load-displacement curve (fig.3). Generally, the load and deformation are linearly related until the *yield region* is reached, at which time the slope of the curve is reduced. Before the yield region, the structure is considered to be in the *elastic region*, and if unloaded, would return to its original shape. The slope of the elastic region of the load-displacement curve represents the extrinsic stiffness or rigidity of the structure (*S*), measured as Young's modulus. The stiffness of the structure indicates how much force is required to deform the structure by a given amount and is defined as the slope of the load-deformation curve in the linear elastic region. However, beyond the yield region, the structure undergoes permanent deformation and is said to be in the *plastic region*. If the load continues to increase, the *failure load or force* is reached, after which the structure fails.

Besides stiffness, several other biomechanical properties can be derived, including the aformentioned ultimate force (*Fu*), work to failure (area under the load-displacement curve, *U*), and ultimate displacement (*du*). Each of these measured parameters reflects a different property of the bone: ultimate force indicates the general integrity of the bone structure; stiffness is closely related to the mineralization of the bone; work to failure is the amount of energy necessary to break the bone; and ultimate displacement is inversely related to the brittleness of the bone.

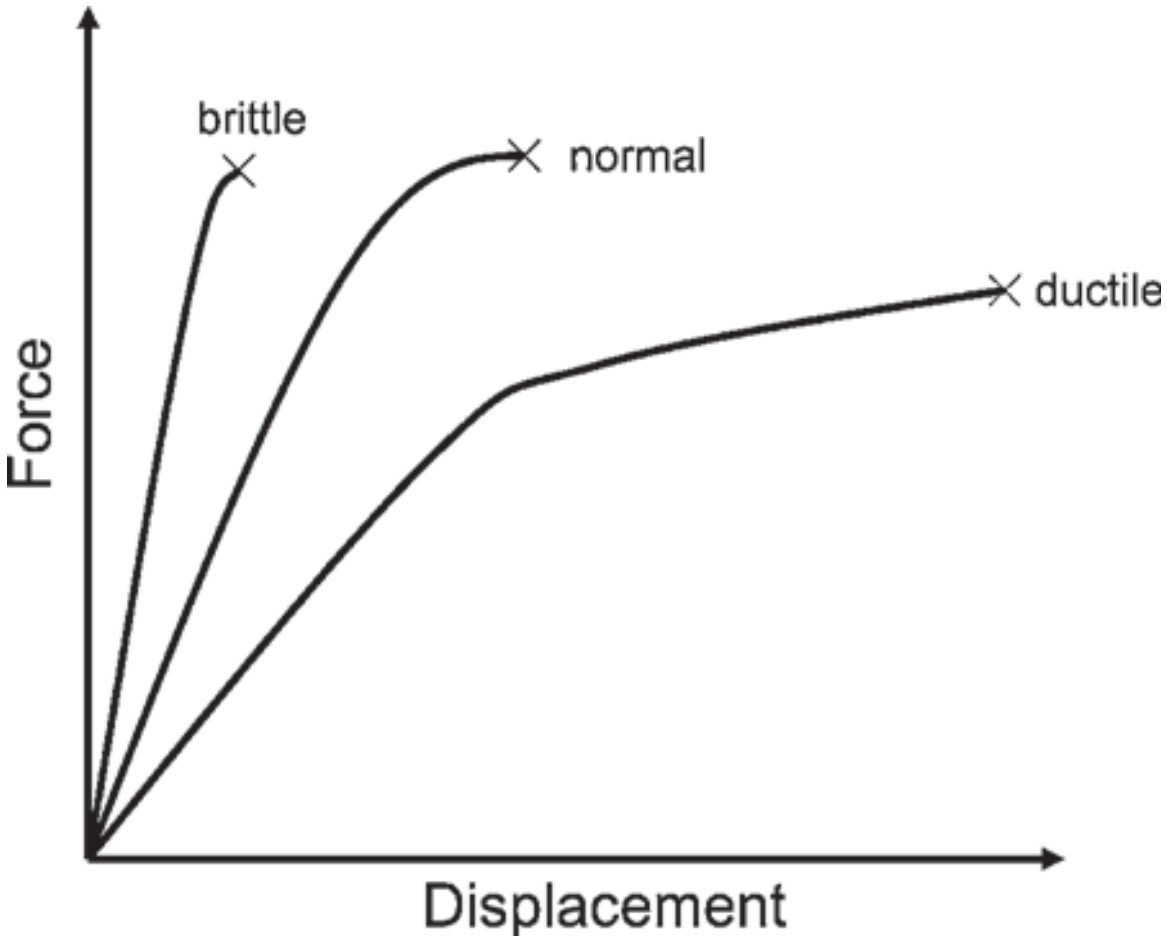
Fig.3 Load-Displacement curve for bone tissue



The biomechanical status of bone may be poorly described by just one of these properties. For instance, a bone from an osteopetrotic patient will tend to be very stiff but also very brittle, resulting in reduced work to failure and increased risk of fracture (fig.4) On the other hand, a bone from a young child will tend to be poorly

mineralized and weak, but very ductile (large ultimate displacement), resulting in increased work to failure.

Fig.4 Load-displacement curves for different bone conditions

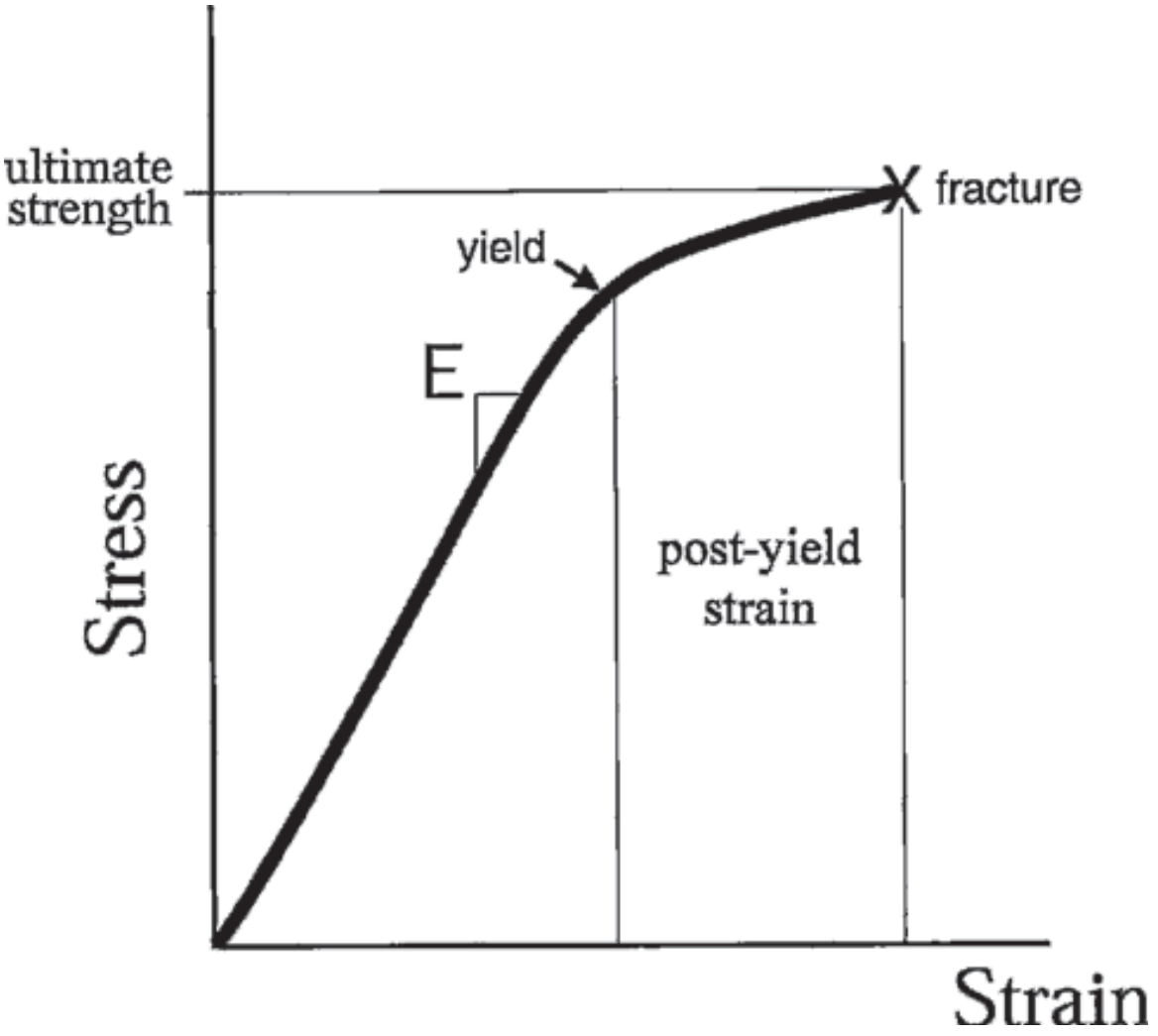


The material properties of a bone specimen are determined using a similar method to that previously described for determining the structural properties. These material properties are derived from a plot of stress versus strain. Stress can be defined as the resistance that develops in response to the applied forces in bone, and it represents the local force intensity with dimensions of force per unit area. The local deformations that result from the applied forces are referred to as strains, which are defined as relative deformations (often expressed in terms of percent).

The relationship between stress and strain in bone follows a curve called the stress–strain curve (fig 5). The slope of the stress–strain curve within the elastic region is called the elastic or *Young's modulus* (*E*). The *Young's modulus* is a measure of the intrinsic stiffness of the material. As the load is increased, the specimen begins to undergo permanent deformation and to yield. If the load is increased further, the specimen will fail. The point of failure is called *strength* or *ultimate stress*. The area under the stress–strain curve is a measure of the amount of energy needed to cause material failure. This property of a material is called energy absorption or *modulus of toughness* or just *toughness*. The maximum stress and strain the bone can sustain are called the *ultimate strength* and *ultimate strain*, respectively.

Strength, as it is defined by the stress–strain curve, is an intrinsic property of bone. That is, these strength values are independent of the size and shape of the bone. In fact, the force required to break the bone is different from the intrinsic strength, because ultimate load will vary with bone size. Hence, intrinsic strength and ultimate load can show different trends in drug or genetic studies, especially if the drug or gene affects the size of the bone. Strength measures that are not presented in units of stress do not represent the intrinsic strength of the material but are influenced by extrinsic factors like specimen size and shape.

Fig.5.Stress-strain curve for bone



The elastic strain region and the plastic strain region of the stress-strain curve are separated by the *yield point* (fig.5). The *yield point* represents a gradual transition, above which stresses begin to cause permanent damage to the bone structure. Post-yield represents permanent deformations of bone structure caused by a slip at cement lines, trabecular microfracture, crack growth, or combinations of these.

Direct measurements of bone biomechanical properties are measured *ex vivo* in the laboratory and can be made on excised bone specimens or whole bones. To

determine the biomechanical properties of cortical and trabecular bone material, standardized specimens are cut from the bone and then subjected to compressive, tensile or shear loads. However in patients, it is not feasible to remove a piece of bone in order to subject it to a biomechanical test in the laboratory. The appearance of non-invasive methods to assess bone strength is essential for a precise study of bone properties and also to correlate these properties with fracture propensity.

1.3.4 Microindentation Technique

Correlations between load-bearing capacity in human cadavers and BMD range from r^2=0.4 to 0.6 (34) . There is clinical and laboratory evidence that, in addition to BMD, the mechanical properties of bone tissue, specifically fracture toughness, play a crucial role in bone strength (35) and would serve as a useful predictor of bone fracture risk. However, clinically available methods for direct estimation of this property require invasive bone sampling (36). Traditionally, measurement of the fracture toughness of bone using methods such as R-curve, J-integral and crack tip opening displacement involves large samples of bones, which obviates their use in clinical practice. In addition, crack growth toughness cannot be determined *in vivo* by employing such techniques.

In the past some attempts have been made to investigate fracture toughness of human bone clinically. Hvid et al developed an “osteopenetrometer” that could be used in the operating room directly over the bone surface (37). Nevertheless, this device used a relatively large indenter tip geometry, over 2mm in diameter, and indented cortical bone by distances in the order of 10mm at force of hundreds of Newtons (37). This highly invasive method proved to be inappropriate for clinical assessment.

In recent years, the value of indentation techniques in the investigation of the mechanical properties of biological materials including bone, dentin and cartilage, has been realized. Nano-indentation techniques for assessing toughness using methods such as Vickers indentation fracture (38-40) (41) are attractive due to their simplicity and their potential to allow for characterization of both local and bulk properties. Using a Vickers indentation instrument, Imbeni *et al*(41). were

able to characterize how cracks propagate and where crack-arrest barriers appear. Vickers indentation testing would, however, be difficult on a living patient because of the need to image, at high resolution, the indentations and the cracks that propagate from the corners of the indentations. Moreover, this imaging can be performed only on bone specimens previously gridded in a way that requires invasive sampling, which again makes the technique non feasible for clinical use. Therefore these techniques are inappropriate for measuring fracture toughness *in vivo*. Using the microindentation technique, the introduction of the reference point indentation (Biodent™ Active Life, inc. Santa Barbara, CA,US) implies a new paradigm for the diagnosis of bone material properties. It is designed to be used without exposing the bone surface.

2. Hypothesis and Objectives

Our hypothesis is that measurement of bone material properties by microindentation is able to discriminate different degrees of bone mechanical strength at the tissue level in fragility fracture cases, controls and in specific conditions of bone strength as clinically observed.

We aimed:

1. to show the feasibility of the technique, and the feasibility of its use in the clinical practice.

2. to test the capacity of the *in vivo* microindentation technique to assess bone material properties in different scenarios: osteoporotic fractures, clinical AFF and LTB treatment.

3. Methods

3.1 Study Populations

Patients included women recruited from the Hospital Universitari del Mar in Barcelona, Spain and, in the study with atypical femoral fractures, patients were also recruited from Hospital Universitario Reina Sofía, Córdoba, Spain.

We included patients with osteoporotic hip fractures without previous treatment for osteoporosis and atypical femoral fractures after long-term bisphosphonates (LTB) therapy, recruited in the acute-care orthopedics ward during the hospitalization following the event.

In the first study we included:

- Patients with typical femoral fractures and

- Patients without fractures or prior treatment as controls.

In the study of atypical femoral fractures we included:

-**Atypical femoral fractures (AFF),** patients who, after long-term bisphophonate therapy (>5 years), met the ASBMR task force criteria (APENDIX 1). A full clinical and radiographic assessment with special focus on the history of bisphophonates (BP) use and other risk factors associated with AFF was obtained.

-**Long-term bisphophonates therapy (LTB)** patients receiving BP therapy (>5 years) and with no history of prevalent fractures.

-**Hip osteoporotic fractures** patients with low-trauma hip osteoporotic fractures without history of prior antiosteoporosis therapy.

-**Controls** patients without fractures or prior therapy.

Exclusion criteria for all groups were previous treatment for osteoporosis (except for AFF and LTB patients) and all-cause secondary osteoporosis (corticosteroids use, a previous diagnosis of advanced renal or liver disease, neoplasia, malabsorption, thyroid or parathyroid disorder, immobilization) or inability to provide consent.

Patients were assessed for bone metabolic disease. Bone mineral density was assessed by DEXA and subclinical vertebral fractures were evaluated by spine Xray. If patients fulfilled the inclusion criteria, signed the informed consent and had no exclusion criteria they were included in the study and a microindentation with Biodent™ (Active Life, Inc. Santa Barbara, CA, US) was performed.

3.2. Reference Point Indentation Testing

All testing was carried out with the Biodent™ reference point indentation instrument (Active Life Scientific Inc., Santa Barbara, CA). Biodent™ is an instrument that uses Reference Point Indentation (RPI) to probe the bone material properties. The probe assembly is comprised of a reference probe and a test probe. The reference probe serves as an anchor against the tissue, and the test probe moves up and down against the sample in a cyclical manner applying an 11N force in order to test bone material properties.

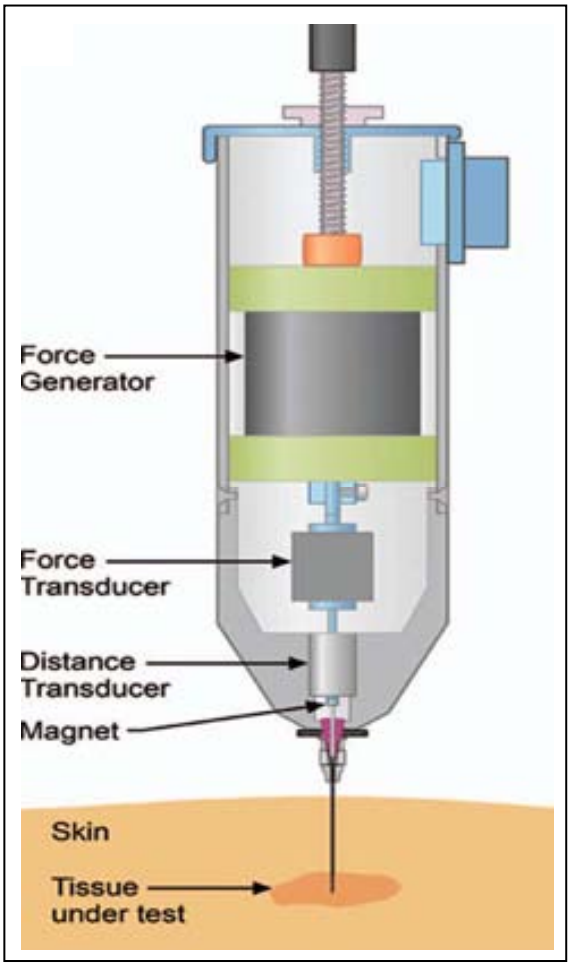


Image 2 Schematic view of Biodent™

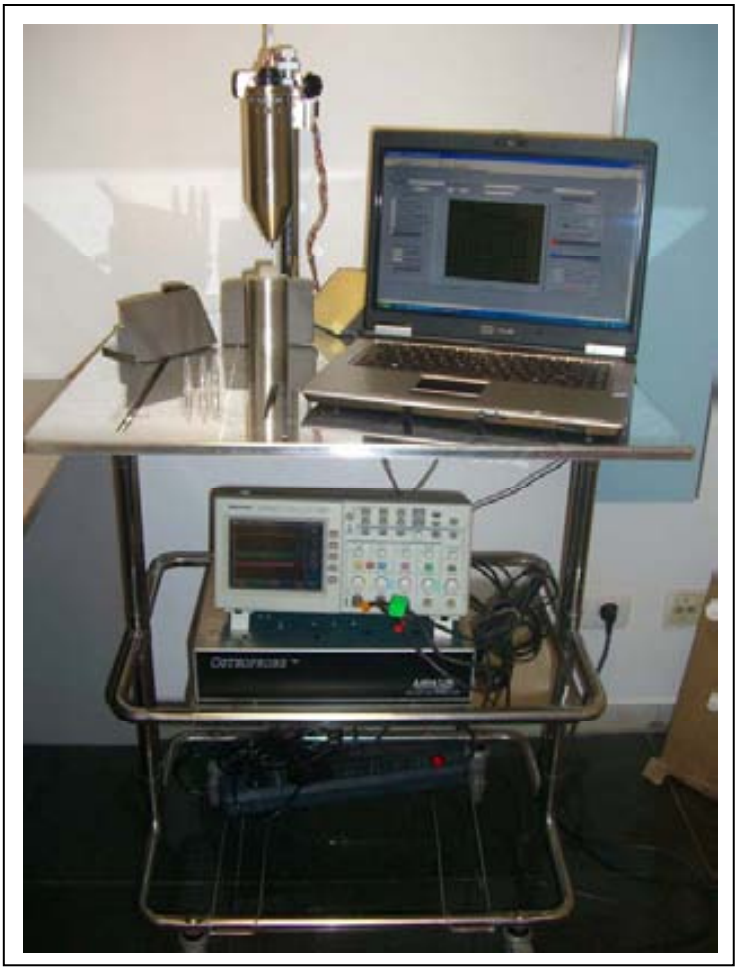


Image 1. Biodent™ (Active Life, Inc. Santa Barbara, CA, US)

To achieve reproducible mechanical testing conditions, patients were placed in a hospital bed with the leg in 15º external rotation.

The Biodent® protocol involves 10 steps:

(1) Attach a presterilized, disposable probe assembly to the head unit of the Biodent™.

(2) Apply clorhexidin and local anesthesia to the testing site (midshaft of anterior tibia). The anterior midshaft of the tibia was chosen for the measurements owing to easy accessibility, as well as offering a relatively

flat surface where the indentation could be made almost perpendicular to the surface.

(3) Use the guidance arm with the vertical slider to position the head unit over the midshaft anterior tibia. The head unit must be perpendicular to the bone's surface within about 15 degrees. Since the head unit is held vertical by the guidance arm with the vertical slider, this is achieved by holding the patient's foot and leg such that the midshaft of the anterior tibia is level to an estimated 15 degrees.

(4) Holding the sterile probe assembly with a sterile glove, lower head unit vertically along slider to insert the probe assembly through the skin to rest on the bone surface.

(5) Displace the periosteum from the measurement area by moving the reference probe by hand laterally along the surface of the bone a distance of approximately 5mm for a series of five times, and then place it in the center of this approximately 5-mm region for measurement. (Periosteum is displaced to avoid interference with the measurements.)

(6) Release the probe assembly so that it rests with the full weight of the head unit on the bone.

(7) Activate the measurement cycle, which first removes an initial 2.5-N force on the test probe (used to keep the test probe from sliding back into the reference probe during insertion) and then begins a series of precycles at 4 Hz that incrementally increase up to a threshold force of the order of 2.5

N and then runs the 20 indentation cycles at 2 Hz each with a maximum force of 11 N.

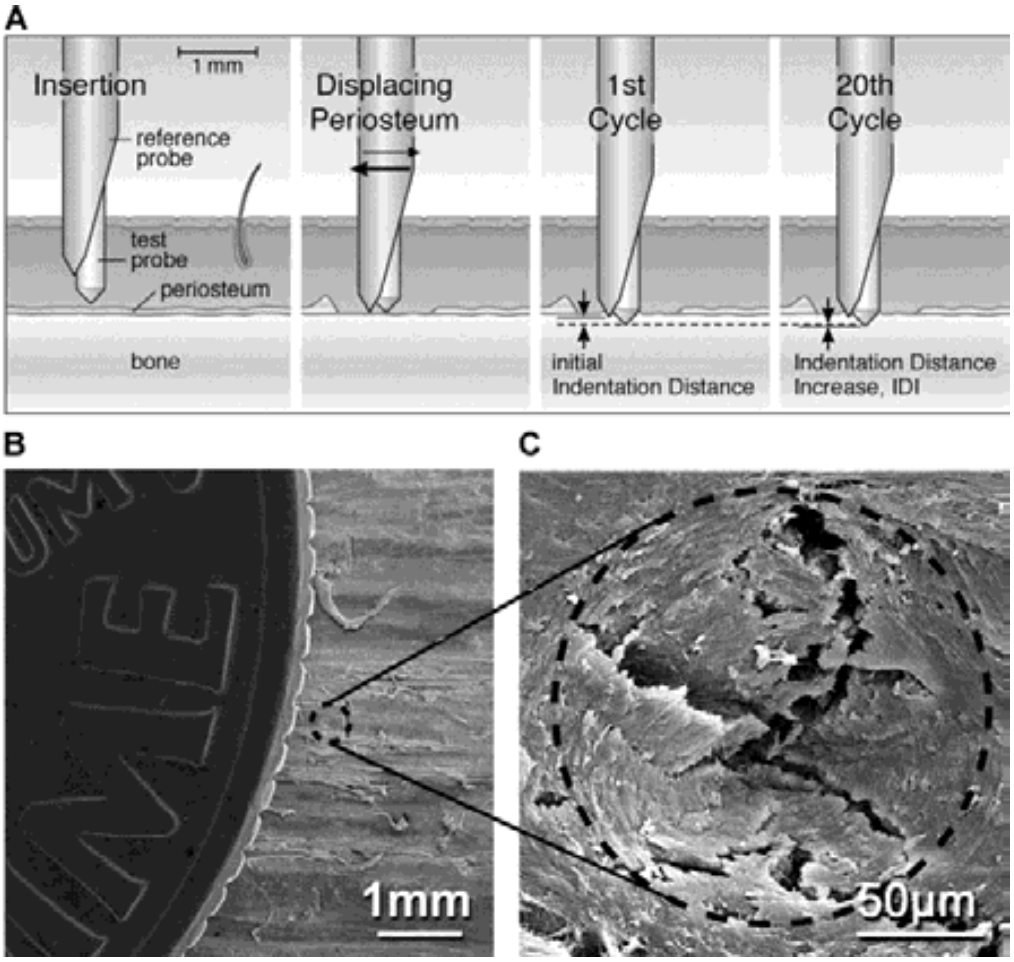
(8) Repeat steps 3 through 7 to obtain measurements at five or more locations. Each measurement location should be separated by at least 2mm from other measurement locations.

(9) After the final measurement, raise the head unit away from tibia, and detach and discard the disposable probe assembly.

(10) Wipe the measurement site with alcohol, and apply a bandage. Local edema or advanced skin disorder and infection in the measurement area would have precluded use of this technique. Warfarin treatment or severe coagulation defects have to be considered for careful local hemostasis. The total cycle time is 500 ms. The purpose of the hold at maximum force is to monitor creep effects and to minimize the effect of the remaining creep during the linear decrease.

Total time for the test is 10 minutes. The patient experiences minimal discomfort (only during the local anesthesia injection), and no complications have been observed whatsoever.

Fig.6 Indentation procedure for measuring material properties of bone in (A) and SEM imaging of an indent on a human bone sample (B and C)



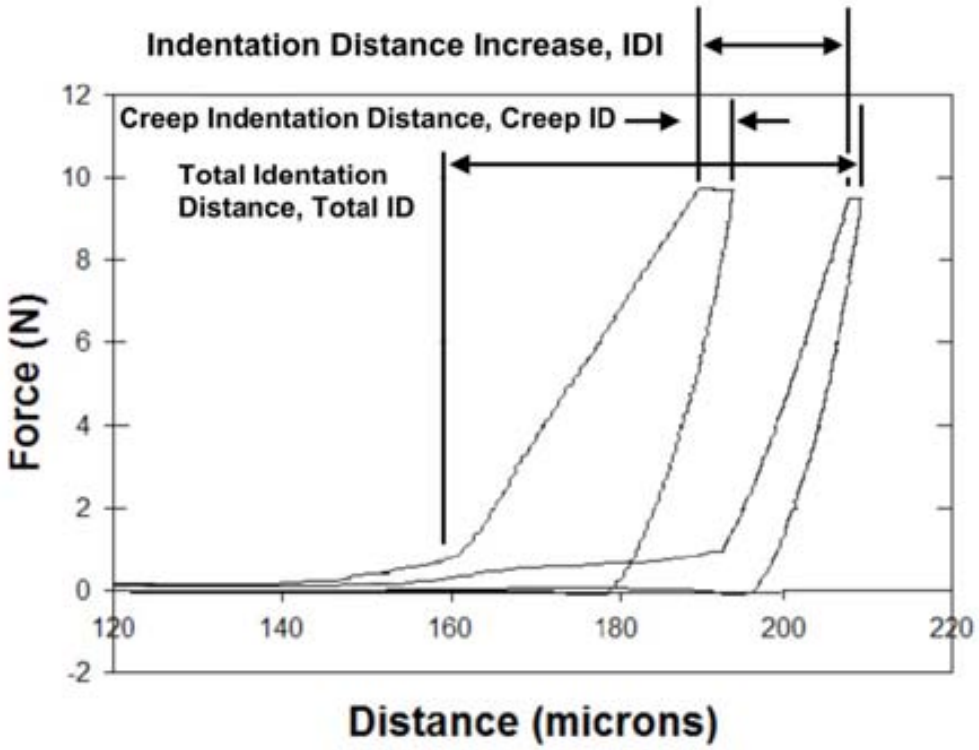
After the cycles are complete, a computer displays the first and last (twentieth) force-versus-distance curves (Fig.7). Three indentation parameters are defined:

Identation Distance Increase (IDI): the difference between the first and the last indentation

Total Indentation Distance (TotalID): the distance after the 20 indentation cycles

Creep Indentation Distance (CreepID): the distance in a 1ms maximum load in the first indentation

Fig.7.Indentation parameters. CORRECT FIGURE: TOTAL INDENTATION DISTANCE



DXA Measurement of BMD

Within 4 weeks of admission, BMD with dual-energy X-ray absorptiometry (using a Hologic QDR 4500 SR Bone Densitometer (Hologic, Inc., Waltham, MA, USA) was measured at the nonfractured hip. In the control or LTB group, DEXA was obtained during outpatients clinic visits.

4.Results and Discussion

4.1 First Study

For our first study we designed a case-control study. We selected patients with typical osteoporotic fractures and patients without fractures. If patients fulfilled inclusion criteria microidentation was performed.

This study involved 27 women with osteoporotic fractures (25 hip fractures and 2 multiple vertebral fractures and 8 controls of comparable age with no fractures.

4.1.1 Results. Mechanical (Reference point indentation- Biodent™) testing

When mechanical testing was performed using Biodent™, two of the three parameters obtained, IDI and TotalD, reached statistical difference. Differences for Creep ID were only marginally significant.

The main clinical data and the microindentation test results are shown in Appendix 2.

In order to accomplish the first objective of this thesis we assessed the interobserver variability doing paired measurements by two observers in the same patient for 14 of the study participants. The coefficient of variation ranged from 8.7% (for IDI) to 15.5% (for totalID).

When measuring the main microindentation variables, the greatest difference between cases and controls was found in the TotalID. Fractured patients had significantly higher TotalID values than controls; IDI also was significantly higher in the fracture group (Appendix 2).

The area under the ROC curve (AUC) value in this study for TotalID was of 0.931 [95% CI 83.1–100], 90.3% for IDI (95% CI 73.2–100), and 73.6% for creep ID (95% CI 56.4–90.9). (Appendix 3)

The correlation with BMD was also studied and, as expected, BMD differences were observed between groups. However, the correlation between total-hip BMD and IDI (r^2 =-.127, p=0.211) and total ID (r^2 =-0.264, p=0.06) was low.

No complications of the technique were detected in any of the 35 study participants.

4.1.2 Discussion

As previously described, the strength of bones is related to mass and geometry, but also to the intrinsic properties of the bone tissue itself. We are able to measure geometry and the mineral content of bone tissue. However, we have not been able until now to measure, directly and *in vivo*, and in a way that is viable for use in clinical practice, the resistance of bone to an impact.

With this first study we showed that *in vivo* microindentation assessed with the Biodent™ instrument is a clinically feasible technique. In the validation process we have developed a suitable measurement protocol and have studied the technique's capacity to discriminate between cases with and without fractures. Interobserver variability also was assessed and resulted in satisfactory values that make feasible cross-sectional interindividual comparisons as well as longitudinal within individual assessments.

The ability to discriminate between cases with and without fracture was demonstrated by finding differences in Total ID and IDI indentation variables

between cases and controls. These variables are directly related to resistance to fracture. These results were consistent with previous reports that greater IDI values were present in brittle bones (Hansma x3 et al and Randall C).

Fracture of bone material can be produced by different techniques. In our case, an indenter produces the fracture and the goal is to determine the fracture toughness.

In fact, fracture mechanics characterizes the tendency of materials to fracture independently of the geometry and even the mineral component. Microindentation allows fracture to be defined as a pure material property, without the effect of the other components that ultimately have influence in fracture resistance of bone, but we were able to measure them directly.

When and how is a fracture produced? The phenomenon of fracture is divided in two parts: crack formation (initiation) and crack propagation. The rate of crack propagation differs by the type of material because it is related to the absorbed energy. If the material is ductile, the crack propagates slowly and the absorption of energy is significant. On the contrary, if the material is brittle, the crack propagates fast and the amount of energy absorbed is low.

Paul Hansma et al. (42) studied with electronic microscopy cadaveric bone samples after microindentation and other samples that were fractured in the laboratory. Both fractured and microindented samples exhibit crack bridging, which resists crack extension.

In the microindentation samples, high IDI values and low crack growth toughness are associated with bones that are prone to fracture (Appendix 4). The BioDent

opened cracks and these cracks are involved in bone fracture. We entertain the hypothesis that propensity to fracture is related to the resistance to crack extension, and thus with the IDI. Lower IDI implies high resistance to crack extension and less probability of fracture.

We can find two scales of indentation: microindentation and nanoindentation. In each case, a load is applied to the indenter that is in contact with the bone and then the load is removed. For microindentation (load range: N), the measurement of the deformed area allows the material properties and the elastic-plastic transition to be determined, which contribute to overall mechanical competence. Microindentation integrates the overall components of bone tissue, both at nano and micro level.

On the other side, nanoindentation (load range: mN) has been utilized to determine the material properties of submicrostructural features of mineralized tissues such as cortical and trabecular bone. However, nanoindentation is performed at the bone structural unit (BSU) level and therefore is restricted to an individual bone package unit with particular conditions of remodeling, age, collagen maturity, and crystallinity.

Both indentation techniques allow us to determine the hardness of a material, defined as the resistance to the penetration of a hard indenter. The main goal of our work was to study bone material properties using microindentation, and test for differences between the two groups studied. The fracture group showed microindentation values that reflected lower resistance to penetration of the indenter and, thus, worse bone material properities.

In line with this result, microindentation appears to measure something different than what is obtained with densitometry. Microindentation also showed better

AUC than BMD, suggesting better ability to discriminate between fracture and control patients.

There was also no significant correlation between BMD and indentation, emphasizing that different parameters of bone properties were studied.

With this first study we established the microindentation technique. A test can be performed in less than 10 minutes, without pain and without complications. We also have validated the technique, showing its ability to discriminate between patients with fracture and controls.

4.2 Second Study

The second study was designed to analyze the bone material properties with microindentation in patients with atypical femoral fractures (Appendix 1) after LTB therapy. The study compared bone material properties measured by microindentation between cases (atypical femoral fractures) and controls (patients without fractures under LTB therapy and hip fracture patients).

4.2.1 Results

The study included 71 women (6 cases of AFF, 38 with typical osteoporotic hip fracture [26 pertrochanteric fractures and 12 subcapital fractures], 6 with LTB use and no incident fracture, and 20 controls).

Baseline characteristiscs and BMD and microidentantion variables of subjects included in the AFF and in the rest of the groups are shown in Table 2 (Appendix 5). Main microindentation characteristics of patients with AFF are shown in table 3 (Appendix 6)

Control patients demonstrate the lowest TotalID values and significantly lower values, indicating better bone material properties than in AFF and typical hip fractures patients.

Along the same line, LTB patients showed similar values of TotalID than the control group but significantly lower values than the typical hip fracture group, indicating better bone material properties than this group. Although no differences were found in TotalID values, the raw values were at a lower order of magnitude than in AFF patients.

Finally, AFF and typical hip fractures showed no differences in the TotalID; raw values had a similar order of magnitude, indicating that these two groups had similar bone material properties.

4.2.2 Discussion

This second study used in vivo microindentation to assess the material properties of bone tissue in patients with atypical fractures after LTB treatment. Atypical femoral fractures have characteristics very distinct from ‘‘typical’’ fragility fractures (43, 44). These occur in the subtrochanteric femur, an anatomical region containing the strongest parts of this bone and unlikely to fracture after a low energy trauma, even in advanced osteoporosis (45). Neither the normal or near-normal BMD nor the cortical thickness observed in most of these patients justify these fractures in such an uncommon region(46-48). Despite the relatively high BMD levels in our AFF cases, bone material properties at the tissue level are similar to that observed in typical osteoporotic fractures. In contrast, patients on LTB without AFF had values similar to nonfracture controls, suggesting that the effect of the drugs on the bone tissue is not negative in the average patient.

Although their values were not statistically different from AFF and typical hip fractures, we believe that this is because of a limited statistical power.

Although the cause of such fractures is unknown(49-52), the fact that BPs decrease bone resorption and formation (53) and also have a long half-life in bone has led to the common belief that atypical fractures associated with their use are due to the marked suppression of bone turnover(54), resulting in the accumulation of microdamage that can lead to fractures (48, 55). However, Jamal et al. (56) provided a dynamic assessment of bone turnover, using tetracycline labeling and performing a biopsy of the femur just below the site of the subtrochanteric fracture. Contrary to most previous reports (57), they did not find evidence of decreased bone turnover in the biopsy specimen. They hypothetize that this may be due to taking the specimen from an area near the fracture site, which was undergoing accelerated remodeling and therefore would not be indicative of the overall rate of bone turnover(58, 59). Moreover, AFF has been observed in patients not treated at all with BP (60) and in monogenic diseases such as pycnodysostosis (61) or hypophosphatasia (62).

Nevertheless, even though the underlying mechanism of AFF is not well understood, the phenotypical characteristics are well established(46). Published initially by Lenart et al (55), patients with AFF showed a unique radiographic pattern, defined as a simple transverse or oblique (≤30°) fracture with beaking of the cortex and diffuse cortical thickening of the proximal femoral shaft. They also have normal or near-normal BMD values. Now

with microindentation we have reported that they also have worse material properties than control patients, and similar to typical hip fracture patients despite being BP therapy. As previously mentioned, the indentation distance is inversely related to the propensity to growth of the crack. Patients with AFF had impaired TotalID values, similar to those observed in patients with typical fractures and significantly different from controls.

Microindentation integrates the overall microstructures of bone. It captures the levels of bone porosity and heterogeneous osteons, the relationship between bone tissue components and increased bone stress, and the interfaces between osteons, microdamage, mineral, collagen, noncollagen proteins, and other components. One measurement assesses the integrated capability of all these elements to dissipate energy in response to a mechanical challenge. As aforementioned, we entertain the hypothesis that microindentation induces the separation of mineralizad collagen fibrils, the intimate mechanism of initiation and propagation of cracks evolving into fracture. Dall'Ara et al demonstrated that *ex vivo* microindentations were able of discriminating between damaged and intact bone tissue extracted from vertebral human bodies (63). They proved the association between microdamage and the reduction of microindentation hardness and stiffness at the bone structural unit level. The ability of microindentation to detect the reduced mechanical properties in damaged bone is probably due to the lower tissue resistance against the local pressure induced.

In this study applying microindentaion we were able to measure bone material properties at a tissue level. These properties were deteriorated in patients with AFF, well beyond what BMD indicated. This deterioration is similar to that for classical fragility fractures of the hip; no significant differences in material

properties measurements were observed between the patients with typical and atypical fracture, but both were significantly different from controls without fracture of the hip, whereas LTB values were generally in between (although not statistically different). Our results suggest that a general, intrinsic effect of BP causing this decrease in tissue properties seems unlikely because this decrease was not observed in patients without AFF after long-term treatment with these drugs. There were trends but not significant differences between patients on LTB therapy and the other groups.

5.Summary

From a mechanical point of view, fractures represent the structural failure of the bone when the forces that are applied exceed the load-bearing capacity of the bone. Until now the test available to assess propensity of bone to fracture was a surogate marker derived from BMD. However, we had no means to assess directly this propensity.

Our work validates a new technique that is clinically suitable to be done *in vivo* and in usual clinical practice that could directly measure bone material properties and, hence, fracture propensity.

The results of these studies are important not only because they demonstrate an association between micro-level bone tissue damage measured by microindentation, but also because this technique has clinical relevance. It is the first time that an indentation technique has been assessed *in vivo*(63) .

We have validated the technique and established a clinically suitable protocol. Microindentation technique discriminated between patients with and without fractures by the differences between them in microindentation parameters. In addition, the microindenter identified deteriorated bone material properties in patients with atypical fractures taking LTB therapies who had near-normal BMD parameters.

This new tool adds new information in the assessment of bone health. Further studies are needed to replicate these results and study different populations, which would allow the technique to be clinically implemented to provide a more accurate evaluation of fracture risk and of bone quality.

At the moment, we have established that microindentation with a known force allows us to directly measure microfracture in the bone surface. We have demonstrated that patients with osteoporotic fractures are better discriminated with microindentation than with BMD. Finally, we detected worse material properties in osteoporotic patients who presented with atypical fractures despite receiving treatment for osteoporosis.

5.1 Conclusions

1. Microindentation technique is a suitable method for the *in vivo* study of bone in a clinical setting.

2. Microindentation is a convenient, painless, minimally invasive and reproducible technique that can be repeatedly performed *in vivo*.

3. Microindentation discriminates between cases of fragility fracture and controls.

4. Discriminant ability of microindentation is excellent and superior to what is described for bone densitometry.

5. At a tissue level, microindentation opens microcracks, the initiation and propagating mechanism of which are the fundamentals of bone fracture.

6. Microindentation is able to detect the profound deterioration of bone material properties in cases of atypical fracture of the femur.

7. Microindentation detects the bone tissue deterioration in patients with AFF while bone densitometry does not.

8. Long-term bisphosphonates exposure does not deteriorate microindentation values.

9. Microindentation has potential to be the best surrogate of fracture propensity because it directly measures the mechanical performance of bone in response to an external force.

5.2 Clinical implications. New Lines of research

Our work has opened new lines of research for our group. It will be important to establish reference values of microindentation measurements in the general population, in order to assess a patient's bone health status by comparison with these values.

To obtain information complementary to BMD , we will also study populations known to have increased fracture risk. These include patients with HIV, diabetes mellitus, and end-stage chronic kidney disease. In line with that, we have begun a new research line in bone disease and HIV infection, where microindentation could improve diagnosis of bone fragility in these patients.

Finally, this technique has the potential for important additional uses in clinical practice. These include follow-up to assess bone material properties in response to osteoporosis treatment or other treatments known to produce bone deterioration, such as as kidney trasplant, corticosteroids therapy, or other bone-toxic drugs. Our team is already doing follow-up of bone material properties in patients starting treatment with corticosteroids, and is working on follow-up of the other treatments as well.

APENDIX 1.
Atypical Femoral Fracture: Major and Minor Features (Shane et al.)

Major features

• Located anywhere along the femur from just distal to the lesser trochanter to just proximal to the supracondylar flare
• Associated with no trauma or minimal trauma, as in a fall from a standing height or less
• Transverse or short oblique configuration
• Noncomminuted
• Complete fractures extend through both cortices and may be associated with a medial spike; incomplete fractures involve only the lateral cortex.

Minor features

• Localized periosteal reaction of the lateral cortex
• Generalized increase in cortical thickness of the diaphysis
• Prodromal symptoms such as dull or aching pain in the groin or thigh
• Bilateral fractures and symptoms
• Delayed healing
• Comorbid conditions (eg, vitamin D deficiency, RA, hypophosphatasia)
• Use of pharmaceutical agents (eg, BPs, GCs, PPIs)

APENDIX 2.

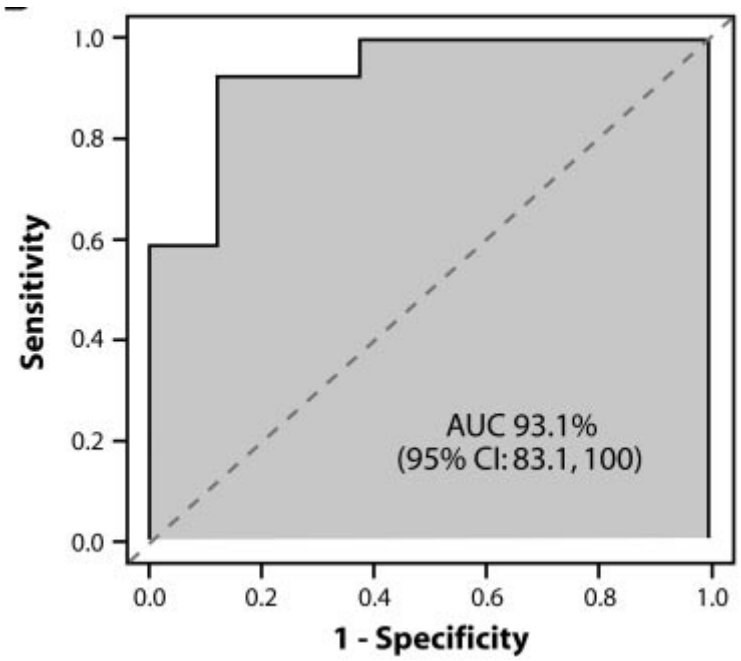
Table 1.Main clinical data and microindentation tests results

	Controls	Cases	p
Age	83.2(5.3)	79.1 (7.5)	0.09
IDI (μm)	12.3(2.9)	18.1 (5.6)	0.008
TotalID (μm)	31.7(3.3)	46 (14)	0.008
CreepID (μm)	3.91(0.6)	5.2 (2)	0.025
FN BMD (g/cm^2)	0.604(0.143)	0.5(0.072)	0.091
Spine BMD (g/cm^2)	0.785(0.163)	0.610(0.101)	0.027

APPENDIX 3.

ROC curve with TotalID.

The area under the curve (AUC) is a scalar quantity to gauge the performance of the curve. An AUC of 100% would represent a perfect model; however, an area going along the line of discrimination (dashed diagonal) would be a completely random model.



APPENDIX 4

SEM images of cadaveric bone samples that were fractured, and exhibit crack bridging, which resists crack extension in a laboratory induced fracture (Cortical and trabecular bone) and . SEM image after microindentation with BioDent™ RPI (right)

APPENDIX 5.

	Control	Long-Term BP (LTB)	Atypical Fractures (AFF)	Typical Fractures
n	20	6	6	38
Age				
Mean (±SD)	69 (±13)	69 (±7) [b]	74 (±6) [a]	82 (±9) [a b]
Range	48-92	58-72	64-84	94-50
Previous Treatment, years (range)	No	5.5 (5-12)	5.4 (5-8)	No
BMD Spine g/cm^2 (±SD)	0.815 (±0.11)	0.734 (±0.11)	0.856 (±0.5)	N/A
BMD Total Hip g/cm^2 (±SD)	0.895 (±0.11) [c]	0.727 (±0.10) [c]	0.848 (±0.10) [d]	0.616 (±0.10)
Total ID	36 (±6)	38 (±4) [b,c]	46 (±4) [e]	47 (±13) [e]
IDI	13 (±2)	16 (±6)	19 (±3) [a]	18 (±5) [a]
Creep ID	4 (±1)	5 (±1)	5 (±0.5)	5 (±2)
25OH Vitamin D ng/ml (±SD)	17 (±9)	36 (±12) [d]	38 (±7) [d]	11.2 (±8)
Ca2+ mg/dl (±SD)	9.3 (±0.5)	9.5 (±0.3)	9.3 (±0.4)	8.5 (±0.6)

Table 2: Main clinical properties and Bone Mineral Density (BMD) results of the enrolled patients (only significant differences shown). Total ID (Total Indentation Distance) IDI (Indentation Distance Increase) Long-Term BP (Long-Term Bisphosphonate treatment). N/A not available

[a] $P<0.05$ vs. controls [b] $P<0.05$ vs. atypical fractures [c] $P<0.05$ vs. typical fractures [d] $P<0.001$ vs. typical fractures [e] $P<0.001$ vs. controls

APPENDIX 6

	Age	BMD Lumbar spine g/cm^2	T-score Lumbar spine	BMD total hip g/cm^2	T-score total hip
Patient 1	77	0.843	-1.9	0.785	-0.50
Patient 2	76	0.921	-1.1	0.962	-0.20
Patient 3	72	0.775	-2.5	0.773	-1.4
Patient 4	64	0.887	-0.30	*f*	*f*
Patient 5	73	0.858	-2.0	0.874	-0.60
Patient 6	84	N/A	N/A	N/A	N/A

Table 2: BMD values in patients with AFF (Atypical Femoral Fracture). BMD values in g/cm^2

f bilateral hip prosthesis replacement

APPENDIX 7

Microindentation for In Vivo Measurement of Bone Tissue Mechanical Properties in Humans

Background

Bone tissue mechanical properties are deemed a key component of bone strength, but their assessment requires invasive procedures. Here we validate a new instrument, a reference point indentation (RPI) instrument, for measuring these tissue properties in vivo.

Patients and Methods

The RPI instrument performs bone microindentation testing (BMT) by inserting a probe assembly through the skin covering the tibia and, after displacing periosteum, applying 20 indentation cycles at 2 Hz each with a maximum force of 11 N.

Results

We assessed 27 women with osteoporosis-related fractures and 8 controls of comparable ages. Measured total indentation distance (46.0±14 versus 31.7±3.3 mm, p=0.008) and indentation distance increase (18.1±5.6 versus 12.3±2.9 mm, p=0.008) were significantly greater in fracture patients than in controls. Areas under the receiver operating characteristic (ROC) curve for the two measurements

were 93.1% (95% confidence intervalo [CI] 83.1–100) and 90.3% (95% CI 73.2–100), respectively. Interobserver coefficient of variation ranged from 8.7% to 15.5%, and the procedure was well tolerated. In a separate study of cadaveric human bone samples (n=5), crack growth toughness and indentation distance increase correlated (r=–0.9036, p=0.018), and scanning electron microscope images of cracks induced by indentation and by experimental fractures were similar.

Conclusions

We conclude that BMT, by inducing microscopic fractures, directly measures bone mechanical properties at the tissue level. The technique is feasible for use in clinics with good reproducibility. It discriminates precisely between patients with and without fragility fracture and may provide clinicians and researchers with a direct in vivo measurement of bone tissue resistance to fracture.

ORIGINAL ARTICLE

JBMR

Microindentation for In Vivo Measurement of Bone Tissue Mechanical Properties in Humans

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ABSTRACT

Bone tissue mechanical properties are deemed a key component of bone strength, but their assessment requires invasive procedures. Here we validate a new instrument, a reference point indentation (RPI) instrument, for measuring these tissue properties in vivo. The RPI instrument performs bone microindentation testing (BMT) by inserting a probe assembly through the skin covering the tibia and, after displacing periosteum, applying 20 indentation cycles at 2 Hz each with a maximum force of 11 N. We assessed 27 women with osteoporosis-related fractures and 8 controls of comparable ages. Measured total indentation distance (46.0 ± 14 versus 31.7 ± 3.3 μm, $p = .008$) and indentation distance increase (18.1 ± 5.6 versus 12.3 ± 2.9 μm, $p = .008$) were significantly greater in fracture patients than in controls. Areas under the receiver operating characteristic (ROC) curve for the two measurements were 93.1% (95% confidence interval [CI] 83.1–100) and 90.3% (95% CI 73.2–100), respectively. Interobserver coefficient of variation ranged from 8.7% to 15.5%, and the procedure was well tolerated. In a separate study of cadaveric human bone samples ($n = 5$), crack growth toughness and indentation distance increase correlated ($r = -0.9036$, $p = .018$), and scanning electron microscope images of cracks induced by indentation and by experimental fractures were similar. We conclude that BMT, by inducing microscopic fractures, directly measures bone mechanical properties at the tissue level. The technique is feasible for use in clinics with good reproducibility. It discriminates precisely between patients with and without fragility fracture and may provide clinicians and researchers with a direct in vivo measurement of bone tissue resistance to fracture. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: BONE; FRACTURE; BONE QUALITY; INSTRUMENT; CLINICAL TRIALS

Introduction

As people age, their bone strength deteriorates, and their bone becomes more susceptible to fracture.[1] The clinical consequence of this, the fracture, contributes to the morbidity and mortality of osteoporosis. Bone strength has been defined as the integration of bone mass and bone quality.[2] Available techniques for clinical estimation of bone strength or susceptibility to fracture are based mainly on bone mineral density (BMD) assessment[3] that can be reliably measured by densitometry techniques, but its sensitivity and specificity are modest.[3,4] Furthermore, its ability to predict the response to a treatment is limited, and only a small proportion of fracture risk reduction is explained by bone density increases.[5] Advanced bone imaging and analysis technologies promise better assessment of bone strength[6] but rely on potentially inaccurate assumptions about the tissue-level mechanical properties. The addition of other surrogates, such as biochemical markers, results in very limited improvement on these strength predictions.[7]

There is clinical and laboratory evidence that in addition to BMD, the mechanical properties of bone tissue may play a critical role in bone strength.[8–10] These mechanical properties would be expected to play a significant role in bone fracture risk, even

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though it has not been clear what mechanical properties are most important.[11–14] However, currently available methods for direct estimates of these properties require invasive bone sampling,[15] making routine use in clinics unfeasible.

Assessment of the intrinsic mechanical properties of bone tissue, as a key component of the widely used concept of bone quality, is limited. Besides the practical inconvenience of their routine measurement, the term *bone quality* is poorly defined and encompasses a series of geometric, microarchitectural, and tissue-composition elements.[15] As a consequence, the potentially relevant contribution of bone tissue strength to fracture risk in clinical practice cannot be evaluated, even though it is known that it deteriorates in osteoporosis and contributes to fracture propensity.[16]

Therefore, there is a critical need to better quantify bone mechanical properties at the tissue level, in particular, the ability of bone to resist the growth of cracks that result in bone fracture. This quantification is not only desirable for more complete clinical assessment of fracture risk but eventually also for treatment monitoring. Moreover, this development could help to better assess the effect of drugs on bone strength without the need for large and expensive prospective fracture trials.

Here we report the validation results of a novel microindentation technique capable of directly testing the mechanical endurance of bone tissue and suitable for a repeated measurement in patients. By measuring indentation distances, we assess the ability of bone to resist crack generation and propagation, the anatomic basis of fracture, in a series of women with osteoporosis-related fractures and controls. Moreover, we have performed exploratory studies on the anatomic substrate of the technique.

Materials and Methods

Subjects

This study involves 27 women with osteoporotic fractures (25 hip fractures and 2 multiple vertebral fractures) measured during the hospitalization following the event in the acute-care orthopedics ward and 8 controls of comparable age with no fractures from the Hospital del Mar, Barcelona, Spain. Fracture patients were excluded if there was some previous treatment with drugs for osteoporosis, corticosteroids use, a previous diagnosis of advanced renal or liver disease, neoplasia, malabsorption, thyroid or parathyroid disorder, immobilization, or inability to provide consent. Exclusion criteria for controls were identical, but in addition, control individuals were required to have no prevalent fracture. Thoracic and lumbar lateral radiographs validated the absence of subclinical vertebral fractures.

Bone microindentation testing (BMT)

The reference point indentation (RPI) instrument (which was called the *tissue diagnostic instrument*[17] and the *bone diagnostic instrument*[18–20] in previous publications) can measure bone mechanical properties, in particular, the resistance to fracture, at the tissue level (Fig. 1*A*). The complete BMT protocol involves 10 steps: (1) Attach a presterilized, disposable probe assembly to the head unit of the RPI instrument.[17] (2) Apply alcohol and local anesthesia to the testing site (midshaft of anterior tibia). (3) Use the guidance arm with the vertical slider to position the head unit over the midshaft anterior tibia. The head unit must be perpendicular to bone's surface within about 15 degrees. Since the head unit is held vertical by the guidance arm with the vertical slider, this is achieved by holding the patient's foot and leg such that the midshaft of the anterior tibia is level to within an estimated 15 degrees or less. (4) Holding the sterile probe assembly with a sterile glove, lower head unit vertically along slider to insert the probe assembly through the skin to rest on the bone surface. (5) Displace the periosteum from the measurement area by moving the reference probe by hand laterally along the surface of the bone a distance of approximately 5 mm for a series of five times, and then place it in the center of this approximately 5-mm region for measurement. (6) Release the probe assembly so that it rests with the full weight of the head unit on the bone. (7) Actuate the measurement cycle, which first removes an initial 2.5-N force on the test probe (used to keep the test probe from sliding back into the reference probe during insertion) and then begins a series of precycles at 4 Hz that incrementally increase up to a threshold force of order 2.5 N and then runs the 20 indentation cycles at 2 Hz each with a maximum force of 11 N. (8) Repeat steps 3 through 7 to obtain measurements at five or more locations. Each measurement location should be separated by at least 2 mm from other measurement locations. (9) After the final measurement, raise the head unit away from tibia, and detach and discard the disposable probe assembly. (10) Wipe the measurement site with alcohol, and apply a bandage. Local edema or advanced skin disorder and infection in the measurement area would have precluded use of this technique. Warfarin treatment or severe coagulation defects have to be considered for careful local hemostasis.

The indentations are small, on the order of 375 μm across (Fig. 1*B*), so they are not harmful to the patient. They are large enough, however, that the bone is fractured (Fig. 1*C*) as the test probe indents the bone. The more easily the bone is fractured, the farther the test probe will indent the bone. Thus we quantify the bone fracture resistance by measuring the indentation distances achieved in a measurement. The indentation has to be performed by the test probe perpendicular to the bone surface, with a tolerance of ±15 degrees to obtain reliable results.

The control system for the reference point indentation instrument supplies a modified triangular wave to its internal force generator for the 20 indentation cycles used in measurements. The modified triangular waveform consists of one-third of a cycle of linear increase, followed by one-third of a cycle hold at maximum force (for measuring creep), and then one-third of a cycle of linear decrease. The total cycle time is 500 ms. The purpose of the hold at maximum force is to monitor creep effects and to minimize the effect of the remaining creep during the linear decrease. After the cycles are complete, a computer displays the first and last (twentieth) force-versus-distance curves (Fig. 2*A*). Three indentation parameters are defined in the figure.

Total time for the test is 10 minutes. The patient experiences minimal discomfort (only during the local anesthesia injection), and no complications have been observed whatsoever.

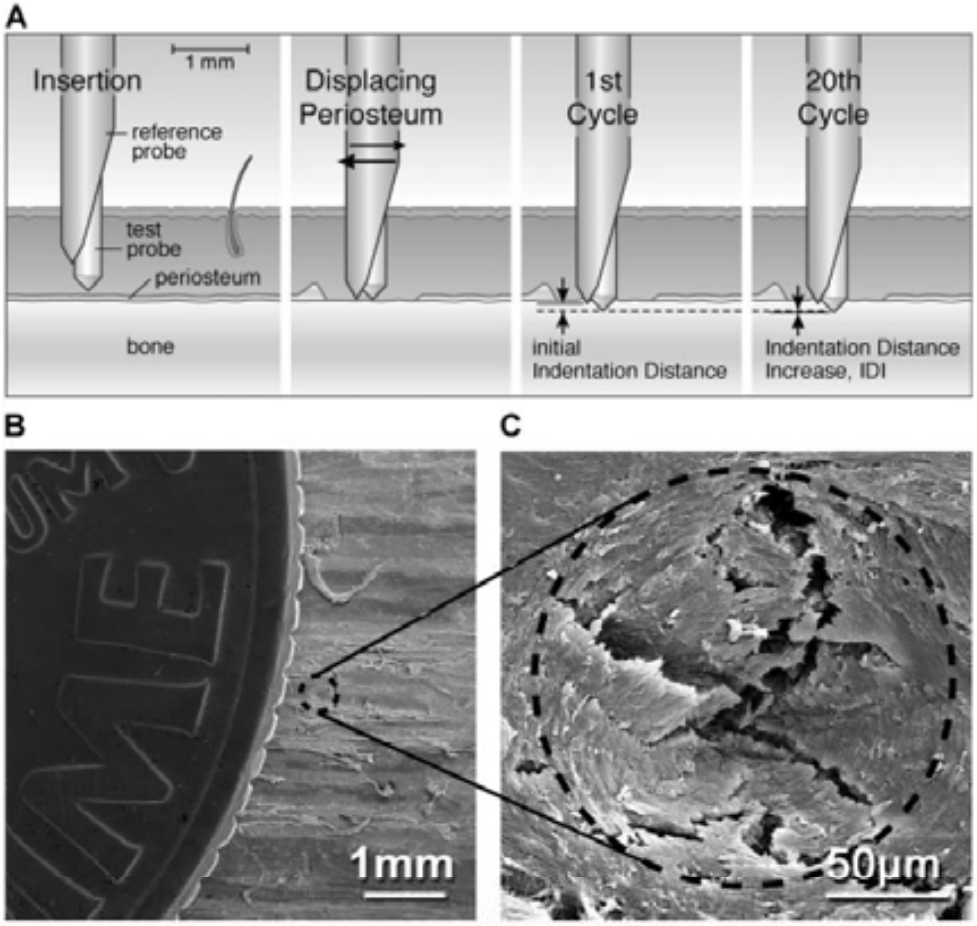

Fig. 1. Indentation procedure for measuring material properties of bone in vivo and SEM imaging of an indent on a human bone sample. (*A*) Illustration of the method for obtaining indentation measurements, including insertion of the test probe assembly, displacing the periosteum with the reference probe, first-cycle indentation, and last-cycle indentation, which determines the IDI with respect to the first cycle. (*B*) SEM image of an indentation (*encircled by dashed line*) being compared to a dime (the smallest U.S. coin). (*C*) This magnified SEM image of the indentation shows microcracks created during the repetitive loading cycles at a constant force.

DXA measurement of BMD

BMD with dual-energy X-ray absorptiometry (DXA) using a Hologic QDR 4500 SR Bone Densitometer (Hologic, Inc., Waltham, MA, USA) was measured at the nonfractured hip within 4 weeks of admission in a subset of 14 individuals randomly chosen (nine fracture cases and five controls) from our clinical cohort.

Statistical analysis

Normality of continuous variables was assessed by Q-Q plots. Analysis of covariance was used to obtain and compare age-adjusted means. Pearson correlation index was computed to assess the relationship between continuous variables. The ability of the indentation distance parameters to discriminate between those who have a fracture and those who do not was assessed by calculating the area under the receiver operating characteristic (ROC) curve.

Preclinical experiments on cadaveric bone

To connect indentation distance increase (IDI), as determined by the reference point indentation instrument, to a conventional measure of fracture resistance on machined samples, we measured both IDI and crack growth toughness on cadaveric bone samples from a group of five donors (aged 17 to 74 years). This is a totally different group from the clinical group discussed earlier. There were eight samples, three for the 74-year-old male, two for the 23-year-old male, and one for each of the other three subjects that gave crack growth data. In the case of the multiple measurements on one donor, the multiple measurements were averaged together to give one data point for the correlation calculation. For IDI data, there were 15 samples, 3 for each donor and 10 tests on each sample for a total of 150 measurements. Again, all measurements on one donor were averaged together to give one data point for the correlation calculation. We were able to do more measurements for the IDI because we could do multiple measurements on each sample, and no special machining was required. The samples were cut from the tibia with dimensions of the order of 2 cm in length and width and the full thickness of the cortical bone. The bone samples were stored in a −80°C freezer. Prior to testing, the samples were brought to room temperature, gently stripped of soft tissue, and placed in Hank's balanced saline physiologic buffer solution[21] to ensure hydration. The surface of the bone

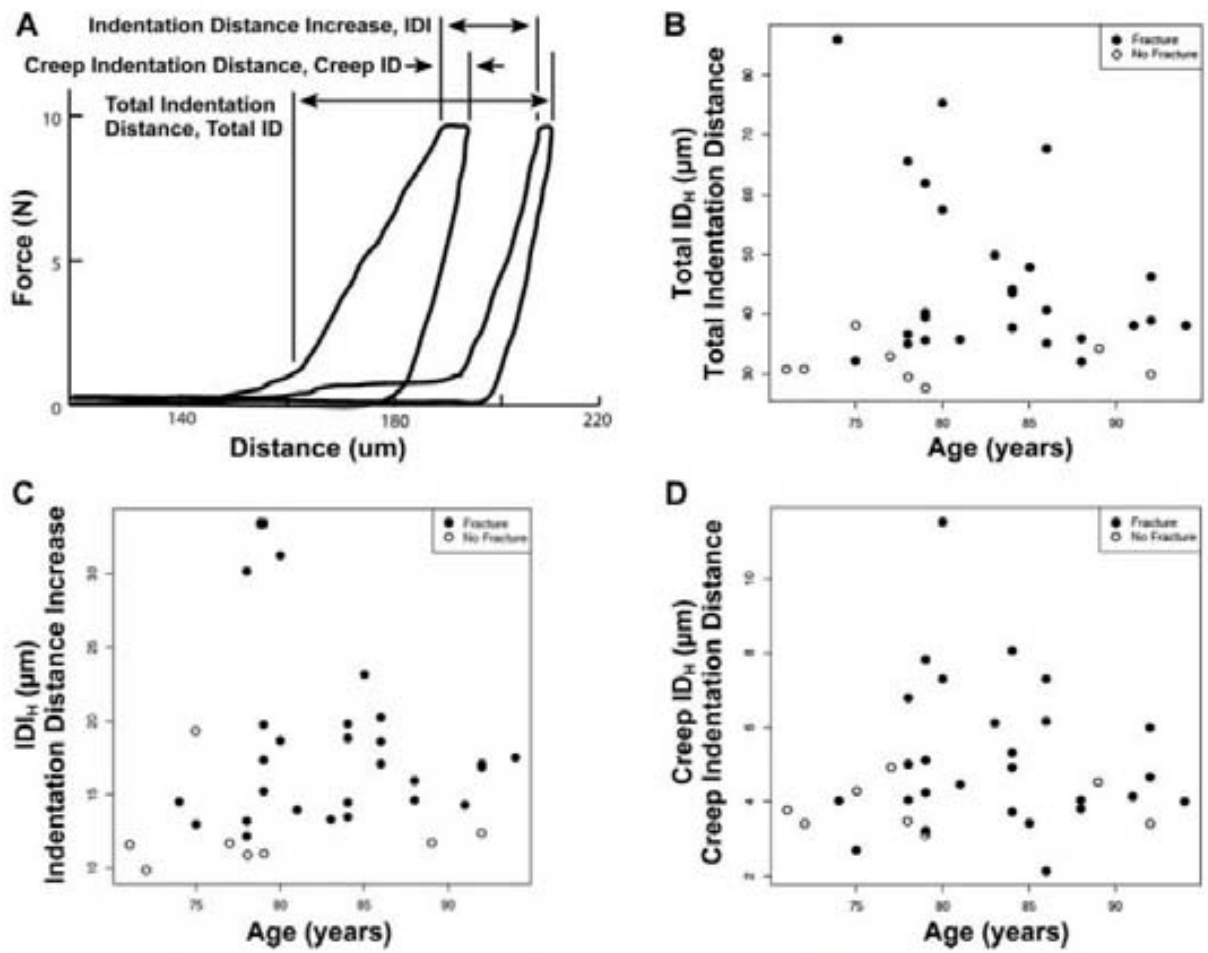

Fig. 2. Parameters are calculated from force-versus-distance data obtained by the RPI instrument. The parameters include indentation distance increase (IDI), total indentation distance (total ID), and creep indentation distance (creep ID) measured in the first cycle. (*A*) The IDI is defined as the increase in the indentation distance in the last cycle relative to the indentation distance in the first cycle (see Fig. 1*A*). The creep ID is determined by the increase in distance while the force is held constant at the maximum value for a duration of one-third of the first indentation cycle. The total ID is defined as the total distance the test probe is inserted into the bone from touchdown to the end of the twentieth cycle. (*B–D*) Results from clinical trials of each parameter with fracture ($n = 27$) and control ($n = 8$) patients. Note that fracture patients usually had higher indentation distances. The subscript *H* on the graphs indicates that the parameters were measured with the Hospital del Mar protocol. This is important because the values of these parameters depend on the measurement protocol.

was not polished. Figure 1*C* shows the microcracks opened by the indentations. Microcracks are opened during RPI testing just as cracks are opened on machined samples during *R*-curve testing. Thus it is reasonable to compare the results of RPI testing with the crack growth toughness from *R*-curve testing.

Indentation testing was conducted by the RPI instrument. The bone samples were held in a vice submerged in physiologic buffer and tested under the buffer. The indentations were normal to the outside surface of the cortical shell. Each sample had a minimum of 10 tests conducted in varying locations. Three samples were tested from each donor. Each individual test was analyzed by software that was written to compute a variety of mechanical parameters such as IDI. The second method used crack resistance curves (*R* curves) to determine the crack growth toughness. Compact tension samples were sectioned and notched transverse to the bones' long axis. The notch orientation was such that the nominal crack growth direction was transverse to the long axis of the tibia. We used nonlinear elastic fracture mechanics testing of the bone samples under hydrated conditions in situ in an environmental scanning electron microscope (ESEM) to permit resistance curve measurements for growing short cracks in the transverse orientation less than 1000 µm in size. Additional details on the testing method and procedure used in this preclinical experiment are discussed by Koester and colleagues.[22] The stress intensity K and crack extension data were linearly extrapolated to determine the growth toughness $\Delta K/\Delta a$ (MPa$\sqrt{}$m/µm), which is obtained from the slope of the R curve.[22-24] Higher growth toughness signifies a bone that is less prone to continued crack propagation.

Results

BMT clinical experiment

Two of the three measured indentation parameters are significantly greater for patients with fractures than for control patients (Figs. 2 and 3). Note also that there is no apparent correlation between age and indentation values, at least in the

A

Variable	Controls (N=8)	Cases (N=27)	p value
IDI (μm)	12.3 (2.9)	18.1 (5.6)	0.008
Creep ID (μm)	3.9 (0.6)	5.2 (2.0)	0.075
Total ID (μm)	31.7 (3.3)	46.0 (14.0)	0.008
FN BMD (g/cm²)	0.604 (0.143)	0.500 (0.072)	0.091
TN BMD (g/cm²)	0.785 (0.163)	0.610 (0.101)	0.027
Age (Years)	83.2 (5.3)	79.1 (7.5)	0.09

B

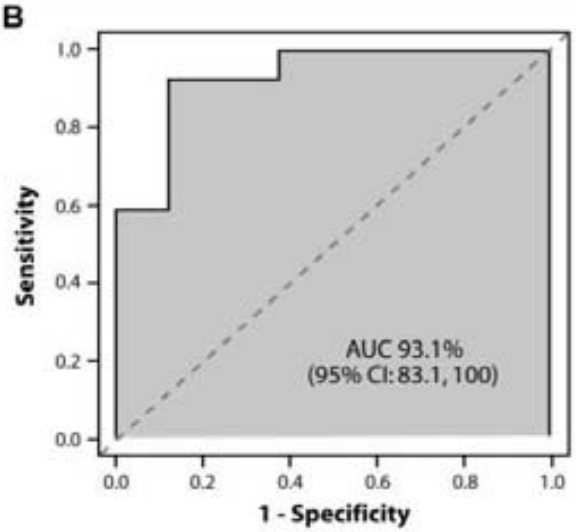

Fig. 3. Data results including statistics and a receiver operating characteristic (ROC) curve. (*A*) Age-adjusted statistical results for IDI (μm), creep ID (μm), total ID (μm), femoral neck bone mineral density (FN BMD, g/cm²), and total-hip bone mineral density (TH BMD, g/cm²). (*B*) The ROC curve displays the clinical results from Hospital Del Mar, Barcelona. The area under the curve (*AUC*) is a scalar quantity to gauge the performance of the curve. An *AUC* of 100% would represent a perfect model; however, an area going along the line of discrimination (*dashed diagonal*) would be a completely random model.

small population of elderly women investigated in this study (Fig. 2). The ROC curve shows that the total indentation distance (total ID) is a good discriminator between patients with and without fractures.[25] The area under the ROC curve (*AUC*) value[26] in this study for total ID was of 0.931 [95% confidence interval (CI) 83.1–100], 90.3% for IDI (95% CI 73.2–100), and 73.6% for creep ID (95% CI 56.4–90.9).

Interobserver variability was assessed by separated measurements performed by two observers in 14 individuals. The coefficient of variation ranged from 8.7% (for IDI) to 15.5% (for total ID).

Differences between cases and controls are shown in Fig. 3*A*. As expected, BMD differences were observed. However, the correlation between total-hip BMD and IDI ($r^2 = -0.127, p = .211$) and total ID ($r^2 = -0.264$, $p = .06$) was low, indicating, as might be expected, that measurements of bone loss (DXA) alone cannot predict bone tissue mechanical properties as measured by the RPI instrument.[25]

Preclinical experiments on cadaveric bone

The results for the comparison between IDI and crack growth toughness are shown in Table 1. The IDI is much greater for the

Table 1. Indentation Distance Increase and Crack Growth Toughness for Each Donor Sample Tested for Correlations

Age/sex	IDI ± SD (μm) (N)	$\Delta K/\Delta a$ (MPa$\sqrt{}$m/μm) (N)
74/M	20.49 ± 6.88 (3)	0.0365 (3)
23/M	14.75 ± 3.12 (3)	0.0428 (2)
17/F	13.97 ± 2.76 (3)	0.0405 (1)
44/F	12.89 ± 3.70 (3)	0.0426 (1)
22/F	12.43 ± 2.49 (3)	0.0455 (1)

Note: The number of samples tested from each donor *n* for each test is shown next to the test result in parentheses. Note the inverse relationship between IDI and $\Delta K/\Delta a$ because high IDI and low $\Delta K/\Delta a$ correspond to a high fracture risk.

74-year-old male subject with an IDI of 20.49 ± 6.88 μm, whereas it is very low for younger subjects. We measured the IDI of cadaveric bone from additional older subjects but were unable to generate an *R* curve for each of the subjects because of the geometry of the bones and the requirements of our testing method.[19] For example, with most of the older individuals who had osteoporosis, there was very little cortical shell to work with on the limited number of samples we had available. Since we had only one older subject from whom we got multiple tests, our results can only be regarded as preliminary. Future testing to compare IDI and crack growth toughness on a wider range of individuals would be valuable. This may require novel methods for determining crack growth toughness.

Figure 4*A–C* shows scanning electron microscope (SEM) images of human bone samples that were fractured and exhibit crack bridging, which resists crack extension. The crack growth toughness of the samples then was compared to the IDI. In samples fractured in fluid,[27] microcracks were observed by SEM, and their appearance was similar to microcracks created by the RPI instrument during repetitive indentations. Comparisons between IDI and the crack growth toughness[22] (slope of the *R* curve) for samples from five donors showed that high IDI and low crack growth toughness are associated with bones that are prone to fracture. The graph shows this trend by relating high IDI to low crack growth toughness and vice versa. Pearson's correlation coefficient between the IDI and crack growth toughness is −0.9036, with $p = .018$ (Fig. 4*D*). The coefficient is negative owing to the inverse relationship between IDI and crack growth toughness.

Discussion

Here we describe the validation study of a novel device that performs bone microindentation testing (BMT) of bone in vivo in a series of patients with and without osteoporotic fractures. BMT discriminates between cases and controls and measures parameters different from BMD. Preclinical studies in human cadavers suggest that BMT induces separation of mineralized collagen fibrils and initiation of cracks, very likely the basic mechanism of fracture, thus directly measuring the mechanical competence of bone tissue to resist fracture.

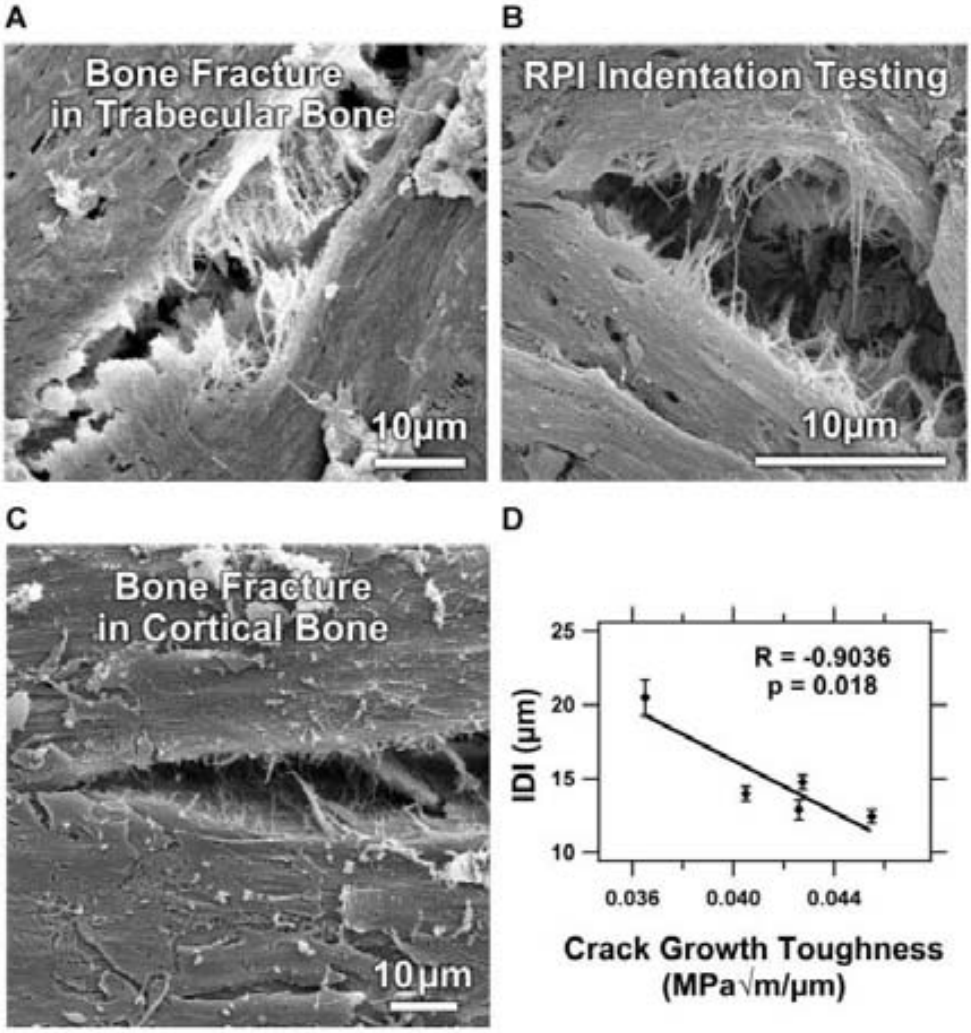

Fig. 4. SEM images of cadaveric human bone samples that were fractured and exhibit crack bridging, which resists crack extension. The crack growth toughness of samples was compared with the indentation distance increase (IDI). (*A–C*) The samples in panels *A* and *C* were fractured in fluid,[27] and microcracks were observed, whereas the sample in panel *B* displays a microcrack created by the RPI instrument during repetitive indentations. It resembles the microcracks in both *A* and *C*. (*D*) Comparison between IDI and crack growth toughness[22] (slope of *R* curve) obtained for samples from five donors. High IDI and low crack growth toughness are associated with bones that are prone to fracture. The graph shows this trend by relating high IDI to low crack growth toughness and vice versa. The linear fit has a Pearson correlation of −0.904, with $p = .018$ (one-tailed) and $p = .035$ (two-tailed). We believe that the one-tailed test is justified because we anticipated the direction of the trend: High IDI corresponds to low crack growth toughness. Because of the limited number of samples and subjects, this correlation should be regarded as preliminary until a more complete investigation is done.

The validation process has followed the usual sequence of developing a suitable measurement protocol and validating the ability of the technique to discriminate between cases with and without the studied condition. Developing the clinical protocol herein described covered the first objective. The anterior midshaft of the tibia was chosen for the measurements owing to easy accessibility, as well as also offering a relatively flat surface where the indentation could be made almost perpendicular to the surface. Periosteum is displaced to avoid interference with the measurements. Interobserver variability also was assessed and resulted in acceptable values that make feasible cross-sectional interindividual as well as longitudinal within-individual comparisons.

The ability to discriminate between cases with and without fracture was demonstrated by the finding of differences in indentation distances between cases and controls. Total ID and IDI showed significant differences, whereas for creep ID, although there was a trend, the difference did not reach significance, very likely owing to the lesser magnitude of this measurement. To further explore this, although the number of cases is limited, the areas under the ROC curve were calculated, yielding excellent values (above 90%) for the two indentation parameters total ID and IDI.

When the BMT values were compared with densitometry measurements in a subset of cases, the differences appeared to be more significant for the former, and the *AUC* values for BMT also were well above the best described for densitometry, even in combined sophisticated assessments.[3–4] Furthermore, there was no significant correlation between the two, further stressing the fact that different parameters of bone properties were studied.

Therefore, tissue mechanical properties, in particular, the resistance to fracture, as quantified by the total ID and the IDI, were significantly different between patients with and without fractures in the clinical results presented here. These clinical results are consistent with six previous laboratory case-control studies in which more easily fractured bone was found to have greater IDI values.[17–20,28] These results can, at least in part, be understood from comparisons of the local microstructure of the cracks opened by the RPI instrument and the cracks involved in bone fracture (Fig. 4). From this study, it appears that as the resistance to crack extension decreases and IDI increases, the probability of fracture increases.

Many possible mechanisms exist that can change the tissue mechanical properties of bone.[13–14,29] These include microcracking[30] and microdamage,[31] changes in mineralization,[12] changes in mineral crystal size,[32] changes in the organic matrix,[33] including posttranslational changes in collagen,[34] changes in collagen fibril orientation,[35–37] and changes in noncollagenous proteins.[38,39] Clinical conditions such as osteogenesis imperfecta further demonstrate the importance of tissue mechanical properties on bone fracture risk. Until now, however, it has been impractical to measure bone material properties in living patients without removing bone samples.

Bone fracture in both trabecular and cortical bone begins with the separation of mineralized collagen fibrils and the initiation of cracks,[38,40–44] as depicted in the SEM images from our laboratory experiments.[27,42,45] The RPI instrument opens cracks that are very similar to those observed following bone fracture. The resistance to extension of the cracks can be quantified, on machined specimens, by resistance-curve (*R*-curve) analysis of the slope of a plot of stress intensity versus crack extension as first shown by Vashishth.[22,24] The slope of the *R* curve is called the *crack growth toughness*, and the larger the crack growth toughness, the larger is the resistance to the extension of cracks. We thus would expect an inverse relationship with IDI, which is smaller if there is more resistance to the extension of the cracks under the tip, as seen in our experiments. This is indeed the case, as demonstrated by the significant negative correlation. This significant correlation relates IDI to crack growth toughness and provides a greater understanding of the physical significance of IDI. This shows that repetitive indentation normal to the bone, as used to determine IDI, is very similar to crack growth toughness; however, IDI can be determined in vivo, whereas crack growth toughness cannot.

There is a substantial history of atomic force microscopy and indentation measurements on bone. A recent review[46] discusses 149 papers. Most commonly, elastic modulus and hardness are measured. Since, however, it is not clear what material parameter (or combination of parameters) best correlates with fracture risk,[11–14] we measured a large number of parameters, including elastic modulus, hardness, initial indentation distance, total indentation distance, indentation distance increase, creep, energy dissipation, and others.[19] From these studies, we discovered that elastic modulus and hardness did not distinguish the bone of patients with and without fractures as well as the parameters reported here, which involved not just one indentation cycle but 20 cycles. It was unclear initially why hardness was a poor indicator of fracture compared with the first-cycle indentation distance because for our tip geometry, a 90-degree cone, hardness is simply the maximum force divided by π times the first-cycle indentation distance squared. The problem was discovered to result from the combined effect of outliers with small indentation distances. They inflated and dominated averages once the raw data of the indentation distance were inverted and squared. Elastic modulus suffered from the same problem, but to a lesser extent. Since elastic modulus depends on the unloading slope after the indentation is made, we were measuring the "elastic modulus" of cracked material, which would not be expected to be characteristic of the uncracked material.

Our BMT technique differs substantially from the previously described osteopenetrometer in several aspects. The osteopenetrometer[47] was developed for intraoperative measurement of bone strength. It used a much larger indenter, over 2 mm in diameter, that indented trabecular bone by distances on the order of 10 mm at forces of hundreds of newtons. These large distances were necessary to average over many trabeculae. Thus the osteopenetrometer is very different from the RPI instrument, which makes microscopic indentations in cortical bone without surgically exposing the bone. The key advance of BMT over previous indentation studies is that the RPI instrument allowed indentation measurements on the bone of living patients without surgically exposing the bone or removing the bone from the patient.

Our study has some limitations. Although we might assume homogeneous mechanical properties of the bone tissue volume unit, our measurements are limited to a cortical compartment and in a given bone, the tibia. Whether this is fully representative of other bones remains speculative at this point, although the primary resistance to mineralized collagen fibril separation might be assumed to be similar across all different skeletal compartments and regions. The number of cases studied is limited, although the differences between cases and controls were strongly significant, which makes a chance finding highly unlikely. Also, our experience is limited to a single center and to a precise group of patients, elderly postmenopausal women. Replication in other groups and populations is warranted.

In summary, we report a novel technique suitable for in vivo measurement of bone tissue strength in a clinical setting. The technique is based on creating microfractures and measuring the overall resistance of bone to the propagation of these microfractures. This represents a direct assessment of bone tissue mechanical strength in patients, an important component of the properties encompassed under the umbrella of "bone quality." Although more research will be needed to use IDI and other parameters measured by the RPI instrument to quantify the contribution of tissue mechanical properties to bone fracture risk, it is already possible to use these parameters to inform the development of novel therapies. This research also opens the possibility of investigations into the differences in the nanoscale fracture mechanisms between bones with different values of IDI.

Disclosures

PH, DB, and AP are members of Active Life Scientific, which sells the Biodent product line of RPI instruments for research use only

at present. If the Biodent or future RPI instruments from Active Life Scientific have a future clinical application, these authors could benefit financially.

Acknowledgments

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APPENDIX 8

Microindentation for In Vivo Measurement of Bone Tissue Material Properties in Atypical Femoral Fracture Patients and Controls

Background

Atypical femoral fractures (AFF) associated with long-term bisphosphonates (LTB) are a growing concern. Their etiology is unknown, but bone material properties might be deteriorated.

Patients and Methods

In an AFF series, we analyzed the bone material properties by microindentation. Four groups of patients were included: 6 AFF, 38 typical osteoporotic fractures, 6 LTB, and 20 controls without fracture. Neither typical osteoporotic fractures nor controls have received any antiosteoporotic medication. A general laboratory workup, bone densitometry by dual-energy X-ray absorptiometry (DXA), and microindentation testing at the tibia were done in all patients. Total indentation distance (Total ID), indentation distance increase (IDI), and creep indentation distance (Creep ID) were measured (microns). Age-adjusted analysis of covariance (ANCOVA) was used for comparisons. Controls were significantly younger than fracture groups.

Results

Bisphosphonate exposure was on average 5.5 years (range 5 to 12 years) for the AFF and 5.4 years (range 5 to 8 years) for the LTB groups. Total ID (microns) showed better material properties (lower Total ID) for controls 36 (±6; mean_SD) than for AFF 46 (±4) and for typical femoral fractures 47 (±13), respectively. Patients on LTB showed values between controls and fractures, 38 (±4), although not significantly different from any of the other three groups. IDI values showed a similar pattern 13 (±2), 16 (±6), 19 (±3), and 18 (±5). After adjusting by age, significant differences were seen between controls and typical ($p<0.001$) and atypical fractures ($p=0.03$) for Total ID and for IDI ($p<0.001$ and $p<0.05$, respectively). There were no differences in Creep ID between groups.

Conclusions

Our data suggest that patients with AFF have a deep deterioration in bone material properties at a tissue level similar to that for the osteoporotic fracture group. The LTB group shows levels that are in between controls and both type of fractures, although not statistically different. These results suggest that bisphosphonate therapy probably does not put the majority of patients at risk for AFF.

ORIGINAL ARTICLE

JBMR

Microindentation for In Vivo Measurement of Bone Tissue Material Properties in Atypical Femoral Fracture Patients and Controls

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ABSTRACT

Atypical femoral fractures (AFF) associated with long-term bisphosphonates (LTB) are a growing concern. Their etiology is unknown, but bone material properties might be deteriorated. In an AFF series, we analyzed the bone material properties by microindentation. Four groups of patients were included: 6 AFF, 38 typical osteoporotic fractures, 6 LTB, and 20 controls without fracture. Neither typical osteoporotic fractures nor controls have received any antiosteoporotic medication. A general laboratory workup, bone densitometry by dual-energy X-ray absorptiometry (DXA), and microindentation testing at the tibia were done in all patients. Total indentation distance (Total ID), indentation distance increase (IDI), and creep indentation distance (Creep ID) were measured (microns). Age-adjusted analysis of covariance (ANCOVA) was used for comparisons. Controls were significantly younger than fracture groups. Bisphosphonate exposure was on average 5.5 years (range 5 to 12 years) for the AFF and 5.4 years (range 5 to 8 years) for the LTB groups. Total ID (microns) showed better material properties (lower Total ID) for controls 36 (±6; mean ± SD) than for AFF 46 (±4) and for typical femoral fractures 47 (±13), respectively. Patients on LTB showed values between controls and fractures, 38 (±4), although not significantly different from any of the other three groups. IDI values showed a similar pattern 13 (±2), 16 (±6), 19 (±3), and 18 (±5). After adjusting by age, significant differences were seen between controls and typical ($p < 0.001$) and atypical fractures ($p = 0.03$) for Total ID and for IDI ($p < 0.001$ and $p < 0.05$, respectively). There were no differences in Creep ID between groups. Our data suggest that patients with AFF have a deep deterioration in bone material properties at a tissue level similar to that for the osteoporotic fracture group. The LTB group shows levels that are in between controls and both type of fractures, although not statistically different. These results suggest that bisphosphonate therapy probably does not put the majority of patients at risk for AFF. © 2013 American Society for Bone and Mineral Research.

KEY WORDS: ATYPICAL FRACTURES; BONE QUALITY; MICROINDENTATION

Introduction

Bisphosphonates (BP) have been available for more than 20 years, possess well-demonstrated antifracture efficacy, and are currently the first-choice osteoporosis treatment. However, some adverse effects have been reported over the past decade, including widespread concern about the incidence of a new type of fracture with characteristic location and radiological appearance in patients on long-term BP (LTB) therapy.[1–10]

These so-called atypical femoral fractures (AFF) have created the increasing perception of potential harm to bone tissue when chronically exposed to these drugs. They do not meet the classic profile of osteoporotic fragility fractures, have been associated with excessive suppression of bone turnover, and affect sites that are not usually affected by osteoporotic fractures, specifically the subtrochanteric femur.[11,12] Moreover, in these patients all the classical parameters to evaluate the fracture risk seem to be unreliable, especially bone mineral density (BMD) measurements. Actually, AFF patients usually show BMD values in the

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osteopenia or even normal range and therefore could not be classified as high risk according to densitometric criteria.[13,14]

One possibility, given the normal amount of bone mineral in these patients, is the presence of a disorder in the intrinsic material properties of bone tissue. The usual thickening of the cortices in these fractures, despite which a fracture occurs, strongly suggests a deterioration of the material properties of bone at a tissue level. Two recent publications show abnormal nanoindentation in cases of long-term bisphosphonate treatment[15] and patients with severely suppressed bone turnover.[16]

Recently introduced is reference point indentation, a new tool that permits direct in vivo measurement of the bone material properties of patients with these fractures, integrating all their components both at nano and micro scale. Moreover, the technique is suitable for clinical use. We have previously tested patients with hip fracture and controls without fracture using in vivo microindentation, a technique that discriminates between fracture cases and controls, yielding an area under the curve (AUC) of 93%,[17] whereas for BMD ranges from 72% to 80%.[18–20] Our current aim is to measure the microindentation values in patients with atypical fractures associated with long-term treatment with BP, comparing their results with those of patients with no fractures, patients on LTB therapy without incident fractures, and patients with "typical" osteoporotic hip fractures.

Materials and Methods

Four study groups were defined and recruited between 2008 and 2011 in the Hospital del Mar, Barcelona, and Hospital Reina Sofia, Cordoba, in Spain. Inclusion criteria for patients in each group are detailed below. Exclusion criteria for all groups were previous treatment for osteoporosis (except for AFF and LTB patients) and all-cause secondary osteoporosis (corticosteroids use, a previous diagnosis of advanced renal or liver disease, neoplasia, malabsorption, thyroid or parathyroid disorder, immobilization) or inability to provide consent.

Atypical femoral fracture

AFF were diagnosed after established clinical criteria: fractures in the area below the lesser trochanter and above the distal metaphyseal flare, with a simple transverse or oblique ($\leq 30^{\circ}$) fracture with breaking of the cortex and diffuse cortical thickening of the proximal femoral shaft without previous trauma.[12,21] A full clinical and radiographic assessment with special focus on the history of BP use and other risk factors associated with AFF[12] was obtained.

Typical hip osteoporotic fracture

Eligible cases were identified from the orthopedic surgery ward with typical hip osteoporotic fracture, which included pertrochanteric fractures and subcapital fractures. These cases had no history of bisphosphonate or any other antiosteoporosis therapy.

Long-term treatment with bisphosphonates and no incident fractures

Outpatients with 5 or more years of treatment with BPs and no incident fractures were included in this group. Thoracic and lumbar lateral radiography validated the absence of incident subclinical vertebral fracture during the treatment period after the index fracture.

Control group of nonfracture individuals

Postmenopausal women who visited our outpatient clinics or were admitted to the Internal Medicine ward for acute illness and who had no history of fractures, no previous use of antiosteoporosis drugs, and no other cause of bone disease constituted the study controls. No previous history of fracture was required, and a thoracic and lumbar lateral X-ray was obtained to confirm the absence of subclinical vertebral fracture.

Bone microindentation testing (BMT)

The BMT was performed using BioDent (ActiveLife Tech, Inc., Santa Barbara, CA, USA). The complete protocol was previously described.[17] In brief, after local anesthesia, the periosteum is scratched and a probe assembly placed on the anterior face of the mid-tibia performs measurements. A 20-cycle indentation at 11N force is performed and the average value of five measurements is recorded. The indentation distances are analyzed by a specific software and three parameters are obtained to use as outcome variables: indentation distance increase (IDI) between the first and last indentation cycle; total distance between the bone surface and the last indentation cycle (Total ID); and creep indentation distance (Creep ID), the progressive indentation distance during the stable force phase of the first indentation cycle at the maximum 11N force.[17] The microindentation testing was done after as soon as possible after the fractures. This was a few days for typical fracture cases. Because of the difficulties in locating and transporting the atypical fracture cases, this was typically a few weeks or months for these cases.

Bone mineral density measurement

Dual-energy X-ray absorptiometry (DXA) with a Hologic QDR 4500 SR Bone Densitometer (Hologic, Inc., Waltham, MA, USA) was used to measure BMD at the nonfractured hip in all atypical fracture cases, in 8 controls, in 9 typical fractures, and in all LTB patients.

Ethical aspects

The Committee on Human Subjects Research at the Municipal Institute of Medical Research (IMIM; Parc de Salut Mar, Barcelona, Spain) approved the study, and written informed consent was obtained from each participant after a full explanation of the purposes and characteristics of the study.

Statistical analysis

Subject characteristics and microindentation variables are expressed as mean (SD) or percentage. Normality of continuous

variables was assessed by Q-Q plots. Analysis of covariance was used to obtain and compare age-adjusted means.

A p value of less than 0.05 was considered to indicate significance. All reported p values are two-sided. The analysis was performed using SPSS for Windows, version 15.0, 2006 (SPSS Inc., Chicago, IL, USA).

Results

The study included 70 women (6 cases of AFF, 38 with typical osteoporotic hip fracture [26 pertrochanteric fractures and 12 subcapital fractures], 6 with LTB use and no incident fracture, and 20 controls). Table 1 shows the main clinical characteristics and BMD results of the enrolled patients. The control individuals without fractures were significantly younger than the women in the fracture groups. The average BP exposure was 5.5 years (range 5 to 12 years) for the AFF group and 5.4 years (range 5 to 8 years) for those with LTB use and no fracture. Among the patients with atypical fracture, all major clinical characteristics defined by the ASBMR task force were present.[12] Four of those six cases also presented minor clinical features (localized periosteal reaction and bilateral symptoms). One patient had sustained a previous contralateral atypical femoral fracture. None of the AFF cases had a history of exposure to glucocorticoids. There were no differences in BMD at the lumbar spine or total hip between the LTB group and controls or AFF, whereas AFF showed significantly higher BMD at the hip than typical osteoporotic fractures.

After adjusting by age, IDI values were significantly different between AFF and controls (19 ± 3 versus 13 ± 2, $p < 0.05$, mean $\pm$ SD) and between typical fractures and controls (18 ± 5 versus 13 ± 2, $p < 0.05$).

Likewise, Total ID values differed significantly between AFF and controls (46 ± 4 versus 36 ± 6, $p < 0.001$) and between typical fractures and controls (47 ± 12 versus 36 ± 6, $p < 0.001$) (Fig. 1 and Table 1).

Among the subgroups of typical osteoporotic fractures, both pertrochanteric fractures and subcapital hip fractures presented no differences in Total ID or in IDI.

Finally, there were no significant differences in the Creep ID value between groups.

In the AFF patients, where DXA was measured, BMD values were in the range of osteopenia at lumbar spine in 5 of the 6 patients and only one was within the limit value for osteoporosis (Table 2). With respect to the four total hip measurements (one patient had had a bilateral hip replacement), three were normal values and one mild osteopenia.

Discussion

The current study used in vivo microindentation to assess the material properties of bone tissue in patients with atypical fractures after long-term treatment with BP. The indentation distance, an inverse estimate of crack growth toughness, was comparable with that observed in patients with severe osteoporosis and typical hip fracture, even though AFF cases had BMD levels in the osteopenia range. In patients without AFF after LTB exposure, the indentation values were between those of fracture groups and controls. To the best of our knowledge, this is the first in vivo study of bone material properties at a tissue level in these groups of patients.

Atypical femoral fractures have characteristics very distinct from "typical" fragility fractures.[22] They occur in the subtrochanteric femur, an anatomical region that contains the strongest parts of this bone and is unlikely to fracture after a low-energy trauma, even in advanced osteoporosis.[23] Their association with LTB has raised the concern of a paradoxical negative effect of these drugs on bone tissue properties.[24–26]

Table 1. Main Clinical Properties and Bone Mineral Density (BMD) Results of the Enrolled Patients (Only Significant Differences Shown)

	Control	Long-term BP (LTB)	Atypical fractures (AFF)	Typical fractures
n	20	6	6	38
Age (years)				
Mean (±SD)	69 (±13)	69 (±7)[a]	74 (±6)[b]	82 (±9)[a,b]
Range	48–92	58–72	64–84	94–50
Previous treatment, years (range)	No	5.5 (5-12)	5.4 (5-8)	No
BMD spine, g/cm^2 (±SD)	0.815 (±0.11)	0.734 (±0.11)	0.856 (±0.5)	N/A
BMD total hip, g/cm^2 (±SD)	0.895 (±0.11)[c]	0.727 (±0.10)[c]	0.848 (±0.10)[d]	0.616 (±0.10)
Total ID	36 (±6)	38 (±4)[a,c]	46 (±4)[e]	47 (±13)[e]
IDI	13 (±2)	16 (±6)	19 (±3)[b]	18 (±5)[b]
Creep ID	4 (±1)	5 (±1)	5 (±0.5)	5 (±2)
25OH vitamin D, ng/ml (±SD)	17 (±9)	36 (±12)[d]	38 (±7)[d]	11.2 (±8)
Ca2+, mg/dL (±SD)	9.3 (±0.5)	9.5 (±0.3)	9.3 (±0.4)	8.5 (±0.6)

Total ID = total indentation distance; IDI = indentation distance increase); long-term BP = long-term bisphosphonate treatment; N/A = not available.
[a] $p < 0.05$ versus atypical fractures.
[b] $p < 0.05$ versus controls.
[c] $p < 0.05$ versus typical fractures.
[d] $p < 0.001$ versus typical fractures.
[e] $p < 0.001$ versus controls.

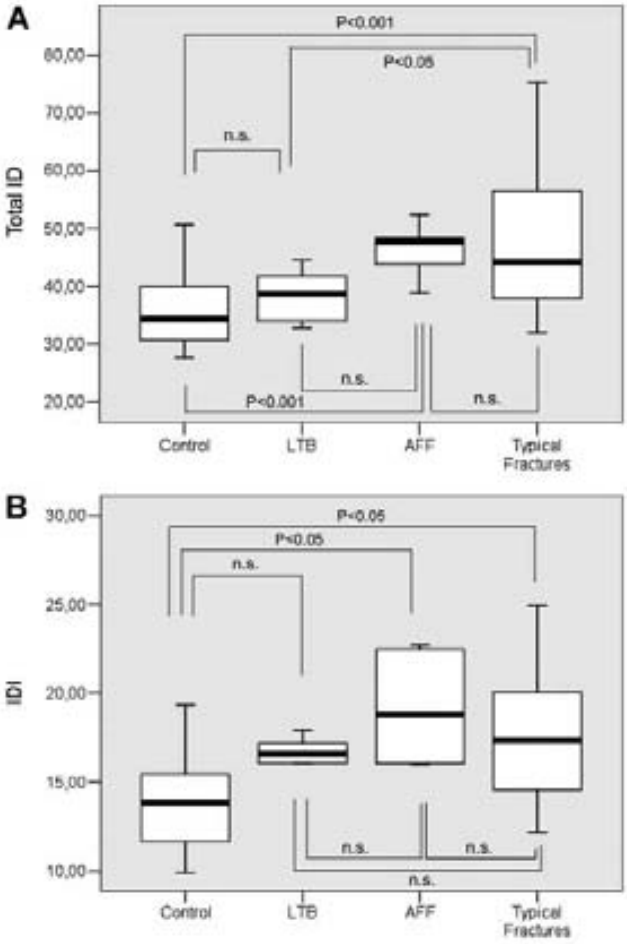

Fig. 1. Microindentation values for the four groups of study subjects. (*A*) Total indentation distance (Total ID), age-adjusted statistical differences. (*B*) Indentation distance increase (IDI), age-adjusted statistical differences. ANCOVA analysis. Boxes indicate the interquartile range and the mean. The bars are the range (lowest and highest values).

The rationale for this deleterious action is that neither the normal or near-normal BMD values nor the thickening of the cortices reported in most of these cases would justify such a severe fracture. Moreover, the healing process is much longer and more problematic than for the typical osteoporotic fracture. Therefore, a negative effect of bisphosphonates on intrinsic properties of the bone tissue (a major component of bone quality) has been strongly suggested.

The obvious question is why all patients treated with BP do not present this impairment in bone properties. The number of patients treated without apparent complication is much larger than the number of observed AFF.[2,3,8,11,27–29] Data from a case-control study showed a prevalence of atypical femoral fractures as low as 1.1% of all femoral fractures and, although more frequent in BP users, they also occurred in patients never treated with BP.[30] Therefore, this problem seems to be restricted to a small minority of treated patients by an undetermined mechanism, although several hypothetical explanations have been suggested.[27]

Nonetheless, the main clinical problem is to clarify if this is a generalized undesirable effect of these drugs on bone tissue material properties or a special idiosyncratic condition in a small subset of patients.[15] Bala and colleagues reported an increased degree of mineralization associated with lower crystallinity in trabecular bone in LTB-treated postmenopausal women. Moreover, microhardness and elastic modulus were decreased by nanoindentation measurement. They conclude that BPs alter the quality of the bone matrix and compromise micromechanical properties. In another study using nanoindentation, Tjhia and colleagues[16] concluded that patients with AFF and severely suppressed bone turnover had greater resistance to plastic deformation at the cortical level.

Our analysis differs from these two studies in several key aspects. We measured the material properties in vivo and in a weight-bearing cortical bone, a more comparable region to where the AFF would occur. More important, microindentation differs from nanoindentation in the measured target. Nanoindentation is performed at the bone structural unit (BSU) level and therefore is restricted to an individual bone package unit with particular conditions of remodeling, age, collagen maturity, and crystallinity. Microindentation integrates the overall components of bone tissue, both at the nano and micro level, which contribute to overall mechanical competence. It captures the levels of bone porosity and heterogeneous osteons, the relationship between bone tissue components and increased bone stress, and the interfaces between osteons, microdamage, mineral, collagen, noncollagen proteins, and other components. Therefore, in a single measurement, microindentation assesses the capability of all these elements to dissipate energy in response to a mechanical challenge.

Accordingly, we entertain the hypothesis that microindentation induces the separation of mineralized collagen fibrils, the intimate mechanism of initiation, and the propagation of cracks evolving to fracture.[17] In fact, when analyzed by electronic microscopy, our technique opens microcracks that can be superimposed on those observed in experimental bone

Table 2. BMD Values in Patients With Atypical Femoral Fracture

	Age (years)	BMD lumbar spine (g/cm^2)	*T*-score lumbar spine	BMD total hip (g/cm^2)	*T*-score total hip
Patient 1	77	0.843	−1.9	0.785	−0.50
Patient 2	76	0.921	−1.1	0.962	−0.20
Patient 3	72	0.775	−2.5	0.773	−1.4
Patient 4	64	0.887	−0.30	*f*	*f*
Patient 5	73	0.858	−2.0	0.874	−0.60
Patient 6	84	N/A	N/A	N/A	N/A

BMD = bone mineral density; *f* = bilateral hip prosthesis replacement; N/A = not available.

fractures;[17,31,32] the more microcracks the indenter opens, the more fragile is the bone and the higher are the indentation values. This effect is much closer to the actual conditions of fracture.

As a result, our data suggest that LTB therapy has a distinct effect in AFF patients. A potential interfering effect of glucocorticoids, usually associated with AFF, plays no role in our patients because they have not been exposed to these components. Despite the relatively high BMD levels in our AFF cases, bone material properties at the tissue level are similar to that observed in typical osteoporotic fractures. In contrast, patients on LTB without AFF are similar to that for nonfracture controls, suggesting that the effect of the drugs on the bone tissue is not negative in the average patient. Although their values were not statistically different from AFF and typical hip fractures, we believe that this is because of a limited statistical power.

Other considerations besides the epidemiological data support the hypothesis that the vast majority of the LTB-treated population is not at increased risk for AFF even after long periods of treatment. The observed cortical thickening has been suggested as a compensatory mechanism opposing bone tissue properties deterioration,[33,34] a phenomenon not typically seen after long treatments with alendronate.[35] Moreover, AFF has been observed in patients not treated at all with BP[36] and in monogenic diseases such as pycnodysostosis.[37] Furthermore, there are no differences between typical and AFF in spite of the fact that AFF cases have BMD values in the range of osteopenia. Considering all these findings, which are consistent with our data, it may be suggested that the group of patients with atypical fractures has some underlying condition of the bone that impairs its material properties, its response to the drug, or both. It has been suggested that the atypical fractures are a phenotype associated with an underlying genetic condition that suffers a clear alteration in material properties under the effect of BP treatment, which leads to these fractures.[38,39]

Our results also have a potential future implication for clinical practice because BMD monitoring does not detect patients on bisphosphonates at risk of AFF. Alerting clinical changes, mainly local pain and, if explored, radiographic or scintigraphic alterations, occur when the bone damage in the subtrochanteric region is already established. Therefore, after 3 to 5 years on treatment, when the question arises of continuing or stopping the therapy, no clinical data are really available to inform medical judgment. If our results are further replicated and prospectively demonstrated, bone material properties testing by microindentation might be a method to decide whether the patient is not showing biomechanical improvement despite BMD increase or if tissue properties are progressively improving, which would reflect a continuous positive effect of the drug that can be further increased.

Our work has both strengths and weaknesses. Microindentation makes a direct measurement of crack growth toughness at the tissue level, is feasible in vivo, and appears to be suitable for clinical use. The technique takes no more than 5 minutes and is reproducible and completely painless. Given that actual microscopic fractures are produced, the technique is supposed to directly assess the fracture propensity of bone. There is no other technique currently available to directly measure the intrinsic bone tissue "quality" without invasive sampling. However, some limitations should be acknowledged. First, the experience with the technique is still very limited and the results, therefore, must be considered as preliminary. Further replication of our results and validation of the value of the technique in other series of AFF, long-term bisphosphonate exposure as well as other clinical situations are needed. Moreover, the low incidence of atypical fractures makes it very difficult to collect a large number of cases. This limited sample size gives a low statistical power and some differences could have been missed, which again raises the need for wider series and replication. Furthermore, we cannot exclude some preexisting idiosyncratic problem in bone material properties in the AFF cases because there are no pre-BP baseline measurements in the treated groups. Similarly, we can only indirectly imply the positive effect of the treatment in our LTB patients because of the lack of baseline assessment.

The very recent development of bone material property testing with microindentation makes some of these limitations unavoidable. Likewise, the practice of microindentation is still limited to a few centers, and wider experience is needed to further validate its performance in clinical assessment. Furthermore, microindentation measurements are obtained in cortical bone, a compartment that only recently has been considered a key factor in fragility fractures;[35] notably, AFF cases suffer a problem precisely located in cortical femur. If cortical bone is affected by LTB treatment, it seems plausible that the tibial measurement would reflect the bone material properties in the subtrochanteric-diaphysis region where the fracture occurs.

In summary, bone material properties at a tissue level, as measured by microindentation, is deteriorated in patients with AFF, well beyond what BMD indicates. This deterioration is similar to that for classical fragility fractures of the hip; no significant differences in material properties measurements were seen between the patients with typical and atypical fracture, but both were significantly different from controls without fracture of the hip, whereas LTB values were generally in between (not statistically different though). Our results suggest that a general, intrinsic effect of BP causing this decrease in tissue properties seems unlikely because this decrease was not observed in patients without AFF after long-term treatment with these drugs. There were trends but not significant differences between patients on long-term bisphosphonates and the other groups. Further studies are needed to understand the paradoxical effect of increased BMD and decreased bone material properties at the tissue level in patients with AFF.

Disclosures

PH is a member of Active Life Scientific, which sells the Biodent product line of RPI instruments for research use only at present. If the Biodent or future RPI instruments from Active Life Scientific have a future clinical application, this author could benefit financially.

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Authors' roles: Study design: ADP and RGF. Study conduct: RGF, ADP, and XN. Patient data collection: LM, ET, JMQ, LP, and RGF. Data analysis: XN, GY, and RG. RGF and ADP performed the technique, and RGF, ADP, XN, NGG, GY, and PKH interpreted the data and discussed the results. Statistical analyses: ADP and RGF. Writing and drafting manuscript: ADP and RGF. Revising the manuscript critically for important intellectual content: XN, LM, ET, LP, and PKH. All authors approved the final version of the manuscript. RGF and ADP take responsibility for the integrity of the data analysis.

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ANNEX

Short Report

"HIV Infection is strongly associated with hip fracture risk, independently of age, gender and co-morbidities: a population-based cohort study"†

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ABSTRACT

HIV infection and anti-retroviral therapies have detrimental effects on bone metabolism, but data on their impact on fracture risk are controversial. We conducted a population-based cohort study to explore the association between clinical diagnosis of HIV infection and hip and major osteoporotic fracture risk.

Data was obtained from the SIDIAP^{Q} Database, which contains clinical information for >2 million patients in Catalonia, Spain (30% of the population). We screened the database to identify participants with a clinical diagnosis of HIV infection, and ascertained incident hip and osteoporotic major fractures in the population aged 40 years or older in 2007-2009. In addition, data on incident fractures involving hospital admission were obtained from the Hospital Admissions database. Cox regression models were used to estimate Hazard Ratios (HRs) for the HIV-infected VS uninfected participants. Models were adjusted for age, gender, body mass index, smoking status, alcohol drinking, oral glucocorticoid use, and co-morbid conditions (Charlson Index).

Among 1,118,156 eligible participants, we identified 2,489 (0.22%) subjects with a diagnosis of HIV/AIDS. Age and gender-adjusted HR for HIV/AIDS were 6.2 [95%CI 3.5-10.9; p<0.001] and 2.7 [2.01-3.5; p<0.001] for hip and major fractures respectively; this remained significant after adjustment for all mentioned potential confounders: HR 4.7 [2.4-9.5; p<0.001] and 1.8 [1.2-2.5; p=0.002]. After stratifying by age, the association between HIV infection and major fractures was attenuated for those aged <59 years (adjusted HR 1.35 [0.88-2.07], p=0.17), but appeared stronger in older patients (adjusted HR 2.11 [1.05-4.22], p=0.035).

We report a strong association between HIV infection and hip fracture incidence, with an almost 5-fold increased risk in the HIV infected, independent of gender, age, smoking, alcohol drinking and co-morbidities. Similarly, we demonstrate a 75% higher risk of all clinical fractures and a 60% increase in risk of non-hip clinical fractures among patients with a diagnosis of HIV infection.

INTRODUCTION

As high activity antiretroviral therapy (HAART) for HIV infection allows patients to live longer, many are being confronted with additional health challenges related to ageing. Morbidities that were not classically considered to be HIV-related are now seen associated with ongoing HIV replication, chronic immune activation, and also with long-term HAART(1, 2). Although potentially severe, osteoporosis and fractures historically have been neglected. Despite the previously established effects of HIV on bone metabolism, the impact of HIV-related bone disease on fracture risk remains uncertain. Numerous studies have found that HIV infected patients have lower bone mineral density (BMD) and higher bone loss rates compared with the general population (3, 4) but studies analyzing whether low bone density actually leads to greater incidence of fractures in HIV infected patients have been inconclusive (4-6). In addition, Collin et al found that the incidence rate of first fractures in HIV-infected patients was in the same range as that reported in the general European population for the same age group(5) .We used a large population-based primary care database to explore the association between HIV infection and the risk of hip, non-hip and all clinical fractures.

METHODS

Study Design: population-based retrospective cohort study.

Participants:

The Spanish public health-care system covers the practical totality of the population. General practitioners (GPs) play an essential role, being responsible for primary health-care, long-term prescriptions and specialist and hospital referrals. The data in this study were obtained from the SIDIAP^{Q} (*Sistema d'Informació per al Desenvolupament de l'Investigació en Atenció Primària-Q*) Database. SIDIAP comprises of electronic medical records of a sample of patients attending GPs in Catalonia (North-East Spain), covering a population of about 5 million patients (80% of the total population) with a total of 3,414 participating GPs. Only data registered by those GPs with the highest scores in coding quality within SIDIAP are included in SIDIAP^{Q}, a higher quality version of the SIDIAP Database, including information on a representative sample of 1.9 million participants (30% of the population of Catalonia)(7). SIDIAP^{Q} comprises the clinical and referral events registered by primary care health professionals in electronic medical records, comprehensive demographic information, prescription and corresponding pharmacy invoicing data, specialist referrals, primary care laboratory test results, hospital admissions, and their major outcomes. Health

professionals gather this information using ICD-10 codes, and structured spreadsheets designed for the collection of variables such as body mass index, smoking and alcohol drinking, blood pressure. Encoding personal identifiers ensures the confidentiality of the information in SIDIAP[Q].

All patients aged ≥40 years old in the database in the period 2007-2009 were eligible for this study (N= 1,118,587). Participants with a clinical diagnosis of HIV infection were identified amongst these using ICD-10 codes (B20, B22 and B24).

Ascertainment of fractures

Clinical fractures registered in the study period (1/1/2007 to 31/12/2009) in SIDIAP[Q] were identified using medical codes for a list of sites of fracture, which are based on the ICD-10 classification (see list of codes used at Appendix 1). Fracture sites considered for these analyses were those defined by Center and Eisman (8) as major fractures (hip, clinical spine, pelvis, tibia, multiple rib, and proximal humerus), and the most prevalent minor osteoporotic fracture in our data (wrist/forearm). Fracture coding has been validated in SIDIAP using both prospective cohort and hospital admission data as a reference: hip, clinical spine and wrist/forearm fracture coding have been shown to be highly specific (99%, 99% and 98% respectively) in SIDIAP(9).

Statistical analyses:

Cox proportional hazard regression models were used to estimate multivariable-adjusted Hazard Ratios (HRs) and 95% CI for the HIV-infected VS uninfected participants. Similar models were fitted for any clinical fracture, hip fracture and non-hip fracture. Age and gender-adjusted and multivariate HRs are reported. The latter were adjusted for the following potential confounders: age, gender, body mass index (BMI), smoking status, alcohol drinking, oral glucocorticoid use, and co-morbid conditions, as listed in the Charlson Index (type 2 diabetes mellitus, diabetic complications, chronic obstructive pulmonary disease, heart failure, myocardial infarction, peripheral vascular disease, cardiovascular disease, chronic renal failure, liver disease, rheumatoid arthritis, paraplegia, gastro/duodenal ulcer, dementia, malignancies and metastatic neoplasm). We then replicated these analyses to look at the effect of HIV on major fracture risk stratified by median age (59 years).

Missing values for smoking status and alcohol drinking were accounted for by addition of a missing category. The validity of the proportional hazards assumption was verified using the Schöenfeld's residuals formal test. All model fitting was carried out using Stata for Mac version 12.

RESULTS

We identified 1,118,156 people aged 40 years or older in SIDIAP^{Q} in 2007-2009. Out of these, 2,489 (0.22%) were either prevalent or incident cases of HIV infection in this same period. HIV infected and uninfected participants were followed up for a median (inter-quartile range) of 2.997 (0.91) and 2.997 (0.001) years respectively. During the study period, 41,907 (3.75%) patients died (178 (7.2%) HIV-infected and 41,729 (3.7%) in the HIV-free population) and 26,126 (2.34%) were lost to follow-up (92 (3.7%) and 26,034 (2.3%) among HIV and non-HIV subpopulations respectively). When compared to the general population in SIDIAP, HIV infected participants were younger (mean (standard deviation) 50.0 (7.6) vs 61.3 (14.2); p<0.001), thinner (BMI 24.5 (4.4) vs 28.4 (4.9); p<0.001), and more likely to be males (75.3% vs 47.8%; p<0.001), current smokers (53.3% vs 18.9%; p<0.001), severe alcohol drinkers (2.7% vs 1.8%; p<0.001), and to suffer from mild (34.3% vs 2.3%; p<0.001) and severe liver disease (0.4% vs 0.1%; p<0.001), and malignancies (3.8% vs 2.9%; p<0.001).

During the study period, 49 and 24,408 clinical fractures (12 and 7,299 hip fractures) were observed in the HIV infected and uninfected patients respectively. Corresponding unadjusted fracture incidence rates were 8.03/1,000 patient-years [95%CI 6.07-10.62] and 7.93/1,000 [7.83-8.03]. Age-specific fracture incidence rates in the HIV infected VS the disease-free participants for all clinical fractures have been plotted [Figure 1]. Hip fracture incidence rates were 2.03 [1.15-3.57] for the HIV infected and 2.37 [2.31-2.42] for HIV free participants.

Age and gender-adjusted Hazard Ratio (HR) for all clinical, non-hip and hip fractures for the HIV-infected patients were 2.67 [2.01-3.53; p<0.001], 2.39 [1.76-3.25; p<0.001] and 6.16 [3.49-10.86; p<0.001] respectively. Fracture risk remained increased for the HIV-infected even after adjustment for potential confounders including BMI, smoking, alcohol drinking, oral corticosteroid use and history of co-morbid conditions: HRs were 1.75 [1.24-2.48; p=0.002], 1.63 [1.12-2.37; p=0.010] and 4.72 [2.35-9.47; p<0.001]. [Table 1] After stratifying by age, the association between HIV infection and major fractures was no longer significant for those aged <59 years (adjusted HR 1.35 [0.88-2.07], p=0.17), but appeared stronger in older patients: adjusted HR 2.11 [1.05-4.22], p=0.035.

DISCUSSION

Key results

We report a very strong association between HIV infection and hip fracture occurrence, with an almost 5-fold increased risk in the HIV infected patients when compared to uninfected participants, independentely of gender, age, smoking, alcohol drinking and co-morbidities. Similarly, we demonstrate a 75% higher risk of all clinical fractures and a 60% increase in risk of non-hip clinical fractures among patients with a diagnosis of HIV infection. These effects were also independent of potential confounders. However, stratified analyses showed that the increase in risk of non-hip major fractures was only significant in older patients (aged 59 years or over), among whom HIV infection appeared related to a more than double risk of fracture.

There is limited prior evidence assessing the relationship between HIV infection and the risk of fragility fractures. Our findings reinforce other studies that have found a positive correlation between HIV infection and fractures (10, 11) . Triant et al. support the hypothesis that HIV infection is associated with an elevated risk of fracture (12) in all fracture sites. However, they did not find differences in hip fractures in women. Arnsten et al.(4) found that HIV infection is independently associated with reduced BMD in a relatively aged cohort of men, and showed that lower BMD was associated with increased fracture risk. Other population-based cohort studies have been carried out to date (10, 11, 13), which also showed an increased fracture risk in HIV-infected patients. However, some of these studies were methodologically different from ours, like the study by Young et al (13) which reported age and gender-indirectly standardized fracture rates from the HOPS cohort. Nevertheless, similarly to our study, there was a higher risk of incident fractures in HIV infected patients within this cohort, even when adjusting by age, sex, substance abuse, comorbidities (13). Conversely, other studies have found no association between HIV infection and fractures (5, 14) , although reduced sample size, and restrictive inclusion criteria might limit the validity of these findings.

Several potential explanations for the association between HIV infection and fragility fractures have been proposed, including a lower bone mass in these patients: a systematic review(15) of twelve cross-sectional studies in HIV infected adults found that the probability of osteopenia and osteoporosis was 6.4 and 3.7 times higher in HIV-infected patients respectively. Pro-inflammatory effects of HIV, including release of cytokines (Interleukins 1,6 and Tumor Necrosis Factor)(16) and HAART side effects on bone metabolism shown in clinical trials (2, 10, 17-19) have been proposed as the causal pathway for the reduced bone mass observed in the HIV-infected. A recent study has reported that the cumulative use of HAART treatments appears independently associated with an

increased risk of osteoporotic fracture(20). In addition, different life-style and hormonal factors that are prevalent among HIV-infected persons could partly account for the increased fracture risk in these patients. Such factors include physical inactivity, decreased intake of calcium and vitamin D, cigarette smoking, alcohol use, opiate use, and low testosterone levels, hepatitis B or C coinfection (5, 21). According to our data, HIV patients have higher prevalence of alcohol consumption, smoking, and viral hepatitis coinfection than the general population.

The main limitation of our study is the lack of individual validation of each one of the fractures observed. However, coding of hip fractures (and other clinical fractures) have been recently validated in the SIDIAP database and shown to be highly specific when compared to prospective cohort studies and to the official national hospital admission database (9). In contrast, fracture coding in SIDIAP has low sensitivity when compared to conventional cohort studies (between 50% and 70% depending on fracture site). Nevertheless, SIDIAP data was completed with hospital-based diagnoses in order to minimise misclassification in this study. If there was still some degree of under-register in our data, this is likely to be at random, and would hence only drive the risk estimates towards the unity. Another limitation of these data is the lack of detailed information on HIV infection (virus load, disease stage, etc) as well as on anti-retroviral therapies used, which are given for free to HIV patients in hospital settings in Spain, and hence do not appear in pharmacy invoice databases. Finally, the low number of HIV infected patients included among the elderly suggests that the age-stratified results should be interpreted with caution, and need replication in external cohorts.

Strengths of our study are the high representativeness and generalizability of the data used: SIDIAP^{Q} covers a representative sample of more than 30% of the total population, and these data are gathered in actual practice conditions. In addition, loss to follow-up is low (<2.5%) when compared to other cohort studies, which limits the possibility of loss to follow-up bias.

In conclusion, we have shown a strong association between HIV infection and hip fracture risk, with more than 5-fold higher risk when compared to the general population. Similarly, HIV-infected patients are at 75% higher risk of major osteoporotic fractures. This is independent of classical fracture risk factors as well as of co-morbidities. The excess risk of non-hip major fractures appears to be highest in older patients.

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TABLES AND FIGURES

Table 1. Hazard ratio (HR) for hip, non-hip and all clinical fractures for HIV infected vs uninfected patients.

	Number of fractures	Fracture IR/1,000 py [95%CI]	Age & Sex-adjusted HR [95%CI]; p-val	Multivariate adjusted HR* [95%CI]; p-val
HIP FRACTURE				
HIV Uninfected	7,299	2.37 [2.31-2.42]	*REF*	*REF*
HIV Infected	12	2.03 [1.15-3.57]	6.16 [3.49-10.86]; p<0.001	4.72 [2.35-9.47]; p<0.001
NON-HIP FRACTURE				
HIV Uninfected	17,839	5.78 [0.70-0.87]	*REF*	*REF*
HIV Infected	41	6.70 [4.93-9.10]	2.39 [1.76-3.25]; p<0.001	1.63 [1.12-2.37]; p=0.010
ALL CLINICAL FRACTURES				
HIV Uninfected	24,408	7.93 [7.83-8.03]	*REF*	*REF*
HIV Infected	49	8.03 [6.07-10.62]	2.67 [2.01-3.53]; p<0.001	1.75 [1.24-2.48]; p=0.002

IR = Incidence Rate; py = person-years at risk

* Further adjusted for body mass index, smoking, alcohol use, oral corticosteroids use, and the following co-morbid conditions (as listed in the Charlson co-morbidity Index): type 2 diabetes, chronic obstructive pulmonary disease [COPD], heart failure, myocardial infarction, rheumatoid arthritis, cardiovascular disease, peripheral vascular disease, renal failure, liver disease, malignancy, paraplegia, ulcer, and dementia.

Figure 1. Age-specific fracture incidence rates (per 100 person-years) in HIV infected vs uninfected patients.

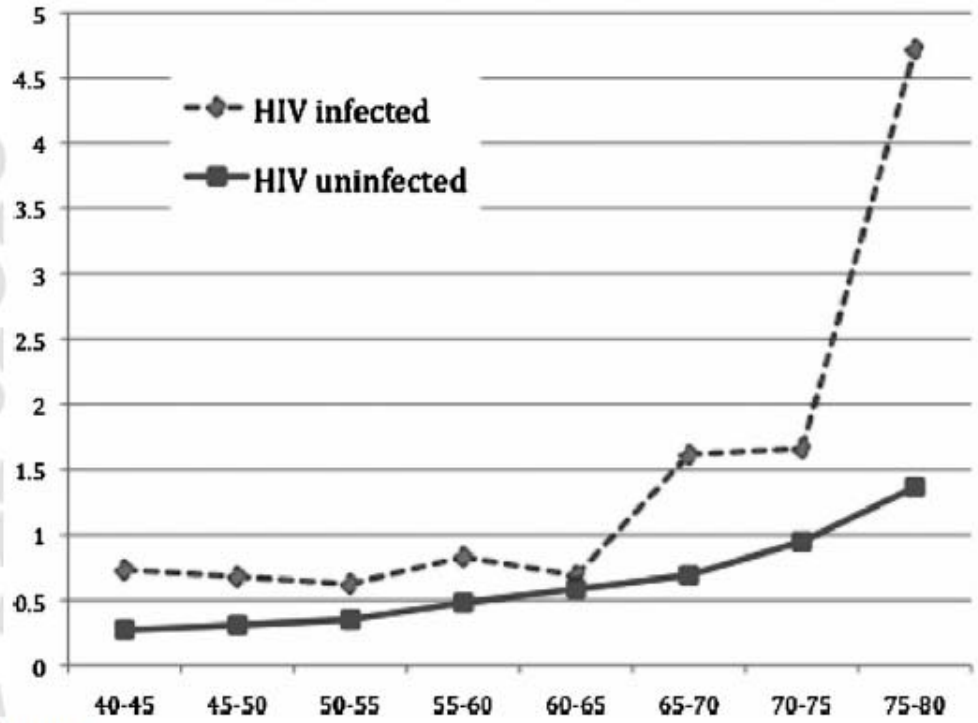

Figure 1

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