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Fourier Transformed Infra-Red Imaging of Femoral Neck Bone: Reduced Heterogeneity of Mineral-to-Matrix and Carbonate-to-Phosphate and more Variable Crystallinity in Treatment-Naïve Fracture Cases compared to Fracture-Free Controls

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Abstract

After age 60 hip fracture risk strongly increases, but only a fifth of this increase is attributable to reduced mineral density (BMD, measured clinically). Changes in bone quality, specifically bone composition as measured by Fourier Transform Infrared spectroscopic imaging (FTIRI), also contribute to fracture risk. Here, FTIRI was applied to study the femoral neck and provide spatially derived information on its mineral and matrix properties in age-matched fractured and non-fractured bones. Whole femoral neck cross sections, divided into quadrants along the neck's axis, from 10 women with hip fracture and 10 cadaveric controls were studied using FTIRI and micro-computed Tomography. Although 3-dimensional micro-CT bone mineral densities were similar, the mineral-to-matrix ratio was reduced in the cases of hip fracture, confirming previous reports. New findings were that the FTIRI microscopic variation (heterogeneity) of the mineral-to-matrix ratio was substantially reduced in the fracture group as was the heterogeneity of the carbonate-to-phosphate ratio. Conversely, the heterogeneity of crystallinity was increased. Increased variation of crystallinity was statistically associated with reduced variation of the carbonate-to-phosphate ratio. Anatomical variation in these properties between the different femoral neck quadrants was reduced in the fracture group compared to controls. While our treatment-naïve patients had reduced rather than increased bending resistance, these changes in heterogeneity associated with hip fracture are in another way comparable to the effects of experimental bisphosphonate therapy, which decreases heterogeneity and other indicators of bone's toughness as a material.

Keywords

Hip fracture; Bone fragility; Mineral/Matrix ratio; Bone heterogeneity; Collagen cross-links maturity; quadrant analysis

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Introduction

Femoral fragility fractures are a significant cause of morbidity and mortality in the elderly and represent a major social and economic burden (1-3). Reduction in BMD, measured clinically at the hip in $\text{g}\cdot\text{cm}^{-2}$, is moderately predictive of who will experience a hip fracture (4), yet 50% of cases have a BMD above the diagnostic threshold for osteoporosis (1). The risk of hip fracture increases as much as ten-fold from age 60 to 80, an increase of which decreasing BMD accounts for only 20% (1). Other important characteristics than BMD, loosely termed 'bone quality', may contribute to bone strength and be independently associated with fracture (5,6). The parameters usually considered to contribute to bone quality are: geometry (7,8); micro-architecture and micro-cracks (their volumetric density and geometric characteristics)(9); cortical bone porosity (10); connectivity of trabeculae (11); and the material properties of the mineralized matrix (12). Material properties depend on mineral and matrix components and the interactions between them (12,13).

Fourier-transformed infrared spectroscopic imaging (FTIRI) has been successfully used to describe the changes in iliac cortical and trabecular bone as functions of age, disease, fracture, and osteoporosis treatment (5,14-17). While average FTIRI properties in the iliac crest are comparable to those in the trochanter and hip, their distributions differ from one tissue to the other (15). FTIRI uses a spectrometer coupled with a light microscope to examine un-decalcified sections of bone at ~ 7 micrometer pixel resolution to provide spatially detailed information concerning mineral and matrix composition. Typically, four key indices are reported: mineral-to-matrix ratio; carbonate-to-phosphate ratio; crystallinity (which is related to the size and perfection of mineral crystals); and cross-link ratio (which is related to the collagen cross-link maturity) (5,14).

Past reports of the effects of diseases and treatments on these FTIRI indices have tended to focus on changes in mean values but sometimes important observations on heterogeneity were made; for example in osteoporosis fluoride treatment is accompanied by increased size of crystals associated with loss of integration with the organic matrix, restricted to newly formed bone (18), while both fracture and bisphosphonate therapy resulted in an increased mineral-to-matrix ratio (16,19) and decreasing heterogeneity (15,20). Furthermore in an assessment of the degree of matrix mineralization based on backscatter electron imaging of iliac crest biopsies from cases of vertebral fractures and controls it was the subject variability rather than the mean mineralization level that was lower in the fracture cases (21).

By assessing the distribution of these parameters across microscopic fields, the level of heterogeneity for selected parameters in the bone tissue can be analyzed. Healthy bone is heterogeneous and there is now evidence that as hypothesized by Keaveny and Hayes (22), this heterogeneity improves bones mechanical properties, perhaps by redistributing mechanical strain levels from the surface, where cracks can initiate comparatively easily, to the less vulnerable interior of trabecular bone (23). Conversely, increasing the homogeneity of these properties might increase bone's brittleness.

The purpose of this study was to test the hypotheses that adverse changes in bone mineral and matrix properties may characterize femoral neck bone in cases of hip fracture. We also wished to examine variations between the anatomical quadrants of the femoral neck because of their different experiences of mechanical loading in the ambulant elderly.

Materials and Methods

Samples and Analyses

Complete, unbroken, femoral neck cross sections from ten women aged 65–91 years were obtained at hemi-arthroplasty following an intracapsular hip fracture due to a fall from standing height or less. Fracture fixation was performed within 48 hours of the fracture. Written informed consent to participate in the study was obtained from all patients. Equivalent material from control necropsies, matched for age (74–89 years) and sex, with no history of disease such as carcinoma or use of drugs known to affect bone metabolism were used as controls. These control subjects died within seven days of admission to hospital; exclusions were cancer, local bone disease and prior immobility. All patients were naïve to bisphosphonate therapy. The quasi-ellipse-shaped femoral neck cross sections (Figure 1) had minor diameters of 30 ± 5 mm and major diameters of 35 ± 5 mm. The thickness (along the femoral neck axis) of the bone samples ranged from 2 to 5 mm.

Each biopsy was fixed with 80% ethanol (EtOH) and slowly dehydrated through a series of increasing concentrations of EtOH, cleared with xylene and finally infiltrated and embedded in polymethylmethacrylate (PMMA) using the Erben method (24). All but one biopsy was evaluated histomorphometrically, after sectioning of the whole specimen. Cortical thickness (25) and porosity (10) was measured in 20 micrometer sections and cortical turnover was evaluated by counting cortical canals that were being eroded, were covered in a layer of osteoid or were quiescent (26). Turnover rates as measured by histomorphometry differed in the anterior portions of the fracture cases and controls (26). The blocks were later orientated, and divided into four quadrants as indicated in Figure 1a to provide four blocks per biopsy.

For the FTIR analysis, 2–3 μm thick sections were cut from each block, normal to the femoral neck axis with a Leica microtome (Leica SM 2500, Leica, Germany) and mounted on barium fluoride infrared windows (SpectraTek, Hopewell Junction, NY, USA). Each biopsy section was examined using a Perkin Elmer Spotlight 300 Infrared Imaging system (Perkin Elmer Instruments, Waltham, MA, USA) at a spectral resolution of 4 cm^{-1} . Images from cortical and trabecular regions of each quadrant as defined in figure 1 were analyzed separately. Background (BaF_2 window only) and PMMA spectra were collected for each section analyzed, and these spectra were used for correction of the sample spectral data. Spectra were baseline corrected and the PMMA spectral contribution subtracted using ISYS software (Spectral Dimensions, Olney, MD) as previously described (27). The following FTIR parameters, reviewed in detail elsewhere (14), were calculated using ISYS software. Mineral-to-matrix ratio, which measures bone mineral content (correlated to ash weight) is calculated from the integrated area of phosphate ($916\text{--}1180\text{ cm}^{-1}$) / Amide I ($1592\text{--}1712\text{ cm}^{-1}$) peaks. The carbonate-to-mineral ratio (C/P), which reflects the level of carbonate substitution in the hydroxyapatite (HA) crystal, is calculated from the ratio of the integrated area of the ν_2 carbonate peak ($840\text{--}892\text{ cm}^{-1}$) and that of the phosphate. Crystallinity, which is related to mineral crystal size and perfection as determined by x-ray diffraction, was calculated as the $1030/1020\text{ cm}^{-1}$ peak intensity ratio. The collagen cross-link network maturity (cross-link ratio) was estimated as the intensity ratio of Amide I sub-bands at 1660 and 1690 cm^{-1} . Heterogeneity in each of these indices was assessed by measuring the full width at half maximum (FWHM) of the pixel histograms. These were calculated from distribution of each parameter in each image for trabecular and cortical bone (20,15).

The residual bone blocks were then used for the micro-computed tomography (microCT). Each quadrant was analyzed both for trabecular and cortical bone by microCT using an eXplore Locus SP micro-CT scanner, (GE Healthcare, London, Ontario, Canada). 80KVp, 0.4 degrees rotation steps (200 degrees angular range), and 2000ms exposure per view were used for the scans of PMMA embedded samples, giving a $28\mu\text{m}$ voxel resolution.

Aluminum 0.02 mm foil was used for reducing the beam hardening effect. MicroView GEHC 2.2 software (GE Healthcare, London, Ontario, Canada) was used for 3D reconstruction and viewing of images as detailed elsewhere (28). After reconstruction, volumes with thickness of 100 μm adjacent to the surface used for FTIRI analysis were segmented using a global threshold of $0.4\text{g}/\text{cm}^3$. Directly measured tissue volume (TV), bone volume (BV), bone volume fraction (BV/TV), total mineral content (TMC) and tissue mineral density (TMD) along with other parameters were calculated for both cortical and trabecular bone, using instrument provided software.

Statistical analysis

Statistical normality of the distributions of residuals, (required for valid linear regression analysis), was assessed by quantile-quantile (Q-Q) probability plots and the Shapiro-Wilk W test. Untransformed distributions were acceptably normal except for the heterogeneities; log transformation was needed to correct the skewness of the distributions (Shapiro Wilk W test p values prior to log-transformation all <0.004). Following transformation, only the cross-link heterogeneities were still significantly ($p<0.05$) positively skewed. Two-way ANOVA was used to assess the effects of anatomical quadrant and disease status (hip fracture case vs. control) on the outcome variables of interest. The micro-CT data were analyzed using SigmaStat (Systat Software Inc, San Jose, CA, USA). Differences between means of the analyzed variables were assessed for each quadrant and between fracture cases and controls. The data were also assessed between trabecular and cortical bone (all quadrants) and between fracture cases and controls. The FTIRI outcome variables (four quadrants each for mean values and heterogeneities: mineral-to-matrix ratio, crystallinity, carbonate-to-phosphate ratio and collagen cross-link ratio) were evaluated separately for cortical and trabecular bone. Each subject was nested within his or her appropriate fracture or control group and within-group variation between subjects was assumed to be statistically random, drawn from either a normal or a log-normal distribution as appropriate. Multiple linear regression models were also constructed, making the outcome variable a function of hip fracture status, anatomical quadrant, and an interaction between fracture status and quadrant. The reproducibility of the results across the three measured regions of interest per quadrant was assessed from the root mean square (RMS) error. When log-transformations were needed, geometric means are reported, while variability is reported as the relative standard error (calculated as % geometric mean) and differences between means are reported as a percentage increase or reduction compared to the control group. Because of multiple testing (4 outcome measures) we set our significance level at $p<0.012$ for each of the FTIRI results (i.e. $p<0.05/4$; 2 tailed test (29)).

Indirect evidence (see e.g. (30)) suggests a relationship between carbonate-to-phosphate ratio and crystallinity in which carbonate may contribute to limiting crystal size. To examine the statistical dependence of mean crystallinity on carbonate-to-phosphate ratio, regression analysis was used with crystallinity as the outcome variable, adjusting for case vs. control status and bone quadrant.

Results

The fracture cases studied and their controls ranged in age from 65-91 years. The histomorphometric analyses of the properties of cortical bone in these femoral neck biopsies are shown in Table 1. These subjects represent a subset of subjects reported on previously using the same methods (10,25,26).

Micro-CT Analyses

Based on micro-CT, the Tissue Mineral Density (TMD) was similar across the four bone quadrants for both the fracture cases and the control (Figure 2a). However, Total Mineral content (TMC) in the fracture cases was significantly lower than in controls in both cortical and trabecular bone ($p < 0.05$) with the inferior region showing a reduction of 26% (Figure 2b). In controls, the inferior quadrant presented higher TMC than the other three quadrants whereas in the fractures these differences were not significant. No differences were observed between fractures and controls for Bone Volume Fraction (BVf) (Figure 2c). Bone Volume (BV) was lower in the fracture group compared to the controls, which was largely attributable to the inferior quadrant that had a 25% reduction in the fracture cases compared to the controls (Figure 2d). All other micro-CT parameters calculated using ASBMR guidelines are summarized in Table 2 and show some significant differences between quadrants in the controls that were not observed in the fracture cases. No significant differences were observed between fracture cases and controls for most of the morphometric bone parameters. Only Tt.Ar in the superior quadrant was significantly lower in fracture cases (-50%) as compared with controls. Both fracture cases and controls presented variation with anatomical quadrant especially at the level of the inferior quadrant. For the cortical bone morphology; the lower mean values for Tt.Ar was observed for the anterior quadrant in the control cases and this anatomical specificity was not seen in fractures cases. Ct.Ar was highest in the inferior quadrant in both control and fracture cases. Inferior and anterior quadrant showed higher Ct.Ar/Tt.Ar values in controls. In both control and fracture cases, the highest values were observed in the inferior quadrant. For the trabecular bone morphology; in the controls the inferior quadrant had the highest values for BV/TV and there were no differences in BV/TV in different quadrants in fracture cases. The inferior quadrant also showed the highest values for Tb.Th in both control and fracture cases.

Effects of Hip Fracture Status and Anatomical Quadrant on mean FTIRI parameters

Typical FTIRI images recorded in the inferior cortical quadrant are illustrated for one patient from the control group (Figure 3a, A) and one patient from the fracture group (Figure 3a, B). These two subjects had comparable TMD and similar ages. The coefficients of variation between regions of the bone images as assessed by RMS errors derived from the best fit models for cortical bone were 5.3, 14.0, 4.4 and 1.9% for mineral-to-matrix ratio, carbonate-to-phosphate ratio, collagen maturity and crystallinity respectively. The equivalent percentages for trabecular bone were 6.7, 16.7, 4.6 and 2.9%. These coefficients of variation were adjusted for mean differences between quadrants.

In cortical bone, the mean mineral-to-matrix ratio was 5.7% lower in the fracture cases compared to the controls ($p = 0.009$). In trabecular bone, the mean mineral-to-matrix ratio was 10.1% lower in the fracture cases compared to the controls ($p = 0.003$ (Figure 3b)). In cortical as well as trabecular bone, in both fracture cases and controls, mineral-to-matrix ratio varied significantly with anatomical quadrant, the highest mean values occurring inferiorly and the lowest, anteriorly (6.2% and 5.8% reductions in anterior compared to inferior, respectively). Mean overall carbonate-to-phosphate ratios and mean overall collagen maturity ratios (Figure 3b) did not differ between fracture cases and controls or between quadrants. Although there were apparent differences between fracture cases and controls in collagen maturity ratios, these were masked by the substantially larger differences between individual subjects within each group, resulting in a large overlap between fracture cases and controls. Collagen maturity ratios were larger in the anterior than the posterior quadrants of trabecular bone and this difference was 4.5% larger in magnitude in the fracture cases compared to the controls ($p = 0.007$).

Mean crystallinity (Figure 3b) showed no significant overall differences between fracture cases and controls in cortical or trabecular bone. However, mean crystallinity varied by anatomical quadrant. In trabecular bone the inferior quadrant had a significantly higher mean crystallinity (2.25% above the overall mean ($p < 0.0001$)) and the same quadrant had higher crystallinity than average by 1.44% in cortical bone ($p < 0.0001$). The posterior quadrant's mean crystallinity values were similar to the inferior quadrant's in the controls, but lower than the inferior quadrant's by ~3% in the fracture cases (p for differences: < 0.011 for trabecular bone; < 0.0001 for cortical bone).

Effects of Hip fracture status and Anatomical Quadrant on Heterogeneity (FWHM)

Heterogeneity of infrared parameters differed when fracture cases and controls were compared. The within-quadrant heterogeneity of mineral-to-matrix ratio was more variable than the mean mineral-to-matrix ratio, with coefficients of variation of 10.2% and 13.5% in cortical and trabecular bone respectively. Overall, the heterogeneity of the mineral-to-matrix ratio was significantly reduced ($p < 0.001$) in the fracture group compared to the controls in both trabecular and cortical bone (by 19 and 16% respectively) (Figure 3c). Between quadrants there was also significant variation in the heterogeneity of the mineral-to-matrix ratio that was comparable in both fracture cases and controls and in trabecular as well as cortical bone ($p = 0.008$ for both). Posteriorly, heterogeneity was 7-10% greater than in the superior or anterior quadrants.

The carbonate-to-phosphate ratio heterogeneity showed posterior quadrant-specific reductions in hip fracture compared to control values (27% and 23% in trabecular and cortical bone respectively). There were no comparable differences in carbonate-to-phosphate ratio heterogeneity in the three other quadrants. Neither were significant differences in heterogeneity seen between fracture cases and controls or between quadrants in collagen maturity ratios ($p > 0.05$) (Figure 3c).

The crystallinity parameter ($1030/1020 \text{ cm}^{-1}$ peak height ratio) showed between-region differences as well as differences between fracture cases and controls. In both trabecular and cortical bone, the heterogeneity of crystallinity was lowest in the inferior quadrant (which also had higher mean values), heterogeneity being only 81% of that seen superiorly in the trabecular bone and 88% of that seen superiorly in the cortical bone. The heterogeneity of crystallinity in the cortical bone also was highest posteriorly (12% higher than superiorly). In cortical bone, but not trabecular bone, there was an additional contrast between fracture cases and controls: fracture cases had a 30% higher heterogeneity of crystallinity posteriorly and a 17% higher heterogeneity inferiorly than controls.

The statistical variations of anatomical quadrant with crystallinity ($p < 0.0001$) and carbonate-to-phosphate ratio ($p = 0.0009$) in controls were significant. Hip fracture had a weaker effect, increasing the overall crystallinity by 8% ($p = 0.015$) through its interactions with anatomical quadrant and carbonate-to-phosphate ratio. Overall, 58% of the heterogeneity in crystallinity was accounted for by these independent variables. Figure 4 shows the associations between mean values for crystallinity and carbonate-to-phosphate ratio in each quadrant for fracture cases and controls. These results suggested the possibility that the heterogeneities of crystallinity and carbonate-to-phosphate ratio might be causally associated.

Because anatomical quadrant has pronounced statistical effects on such properties as cortical thickness, porosity and remodeling, we questioned whether each quadrant was acting as a surrogate for structural strength or remodeling activity. We also asked whether the differences between cortical and trabecular bone reflected the much greater "porosity" of trabecular bone. The effects of the structural and remodeling indices listed in Table 1 on the

means of the four FTIRI outcome measures were not statistically significant ($p>0.05$). However, increasing cortical porosity did have a marginally significant statistical effect to reduce the heterogeneity of the mineral-to-matrix ratio ($p=0.049$), which was independent of the reduction in heterogeneity also associated with hip fracture.

In a model of the determinants of the log-transformed heterogeneities that included quadrant and fracture status, trabecular bone had generally higher heterogeneities of all the FTIRI derived measurements compared to cortical bone. There was overall a 32% higher heterogeneity of the mineral-to-matrix ratio ($p<0.001$) and a 17% higher heterogeneity of the carbonate-to-phosphate ratio ($p<0.001$), an 11% higher heterogeneity of the collagen cross-links ratios ($p<0.001$) and a 32% higher heterogeneity of the crystallinity ($p<0.0001$) compared to cortical bone. These differences between cortical and trabecular bone were not significantly affected by fracture history or by anatomical quadrant ($p>0.05$).

Discussion

In this study we found several bone FTIR spectroscopic imaging compositional properties associated with femoral neck fracture. Mineral-to-matrix ratio, previously reported (31,32), was generally reduced in fracture cases compared to controls. Otherwise, the differences found, were in the heterogeneity of these FTIRI properties. In the herein studied fracture cases there was reduced heterogeneity (i.e. microscopic variation) in the mineral-to-matrix and carbonate-to-phosphate ratios amounting to more than 15% and 20% respectively in all quadrants, measured in cortical or trabecular bone.

Cracks in bone may initiate at locations of increased stress, e.g., where there is discontinuity such as an osteocyte lacuna or a Howship' lacuna (33). The growth of cracks is resisted by processes that increase energy absorption during fracture (toughening mechanisms). The effect of length scale in determining the contribution of toughening mechanisms to fracture resistance, was investigated by Zimmermann et al. (34) who found that increased mean mineralization levels are associated with reduced toughness at the micrometer scale, however, sub-micrometer scale loss of toughness was associated with non-enzymatic collagen cross-linking. The importance of length scale to the effects of toughening mechanisms may also be considered in relation to the development of cracks, which in a tough material like normal bone are generally restricted in length. As a crack develops it tends to accelerate, a process inhibited by toughening mechanisms that absorb the energy of the crack. Failure of "crack capture" by sub-micrometer mechanisms require that other mechanisms, capable of absorbing considerably more energy at micrometer scales, be present, to prevent the crack from developing into a clinical fracture. At the scale level of the whole cancellous bony trabeculae, there is uncertainty as to how inhomogeneity in calcium content influences overall toughness. Inhomogeneity was associated with increased shear stresses (35). Similarly, a finite element modeling study by Renders et al. (23) suggests that inhomogeneity also internalizes these stresses. This process is likely to help protect the trabeculae from mechanical failure, by diverting crack-derived stresses away from surface stress-enhancers such as osteoclastic resorption lacunae.

While FTIRI technology enables us to assess FTIR properties of an individual pixel with an area of $39 \times 10^{-6} \text{ mm}^2$, heterogeneity, was measured across areas slightly under 0.2 mm^2 , considerably larger than the cross sectional area of normal osteons, typically 20-fold smaller. Reduced mean mineral density in hip fracture cases, found in our previous studies, was based on different individual subjects from the same cohorts (31,32). There were no comparable reductions in modulus (32) which might be relevant to the toughness of bone at multiple scale lengths. Our results indicating FTIRI heterogeneity are principal to the study of mechanisms opposing the largest cracks that escape capture at the sub-micrometer scale.

The rate of normal remodeling in the femoral neck is roughly ten-fold lower than that in iliac trabecular bone (36). An explanation for decreased heterogeneity of mineral content in fracture cases is a less actively remodeled tissue, compared to controls, possibly associated with reduced osteocyte density adjacent to the canal (37). The proportion of cortical osteons undergoing remodeling is not reduced in cases of hip fracture. This implies that there might be a longer formative phase in each remodeling osteon, potentially driven by increased late-phase sclerostin expression (38). An alternative explanation, are the physio-chemical differences (i.e., lower levels of calcium and/or phosphate) in the bone micro-environment. The much higher turnover in the iliac crest than in the femoral neck bone might explain why Ciarelli et al found increased mineralization heterogeneity in iliac bone from osteoporotic vertebral fracture cases (21) in contrast to our results. Ciarelli et al later found that this increased iliac heterogeneity was attributable to variations in mineralization between different BMUs according to whether they were recently formed or were older (39). Ciarelli et al similarly found reductions in differences between the mineralization levels in adjacent light and dark lamellae in some categories of osteoporosis (40). Present resolution of FTIRI technology (~7µm) is unable to detect differing mineral-to-matrix ratios between adjacent lamellae. We cannot, as yet, explain the discrepancy between our results and those of Bousson et al (41), who found increased heterogeneity of mineralization in fractured inferior femoral neck bone using microradiography.

Micro-CT data showed a slight but not significantly reduced mineral density in the fracture group. Relative differences in mineral density between fracture and non-fracture groups were smaller than estimated by FTIRI in this study, and by BSE Imaging in previous studies (31,32). These differences were likely due, in part, to the micro-CT partial volume effect, which can be substantial in analysis of the outermost 100 µm of a cortex (42) (as little as 300 µm thick (43)). Moreover, with FTIR spectroscopy, the mineral content is assessed by the mineral-to-matrix ratio. This includes all tissues present, and cannot be directly compared to TMD or BMD, which only consider those regions where the chosen density threshold defines the presence of mineral. These results highlight the importance of both the mineral and the organic matrix in contributing to bone strength (44). The reader should be cognizant of the limitations of micro-CT methods when variance in organic matrix composition may be involved.

Crystallinity as well as mineral-to-matrix ratio was most homogeneous in the inferior quadrant, while the heterogeneity of crystallinity was increased in the posterior quadrant in the hip fracture cases. It is generally accepted that in standing and walking, the inferior quadrant undergoes compression loading, while the posterior quadrant lies across the neutral axis (45) where the bone is neither compressed nor under tension during stance. However in a sideways fall, compression loading is maximal postero-superiorly, a quadrant that is lightly loaded during walking (45).

Biological hydroxyapatite is a carbonate-substituted apatite of low crystallinity (44). Carbonate substitution influences bone properties; increases bone solubility (44), increases with aging (5,46,47) and locally regulated by osteocalcin (48). High carbonate-substitution in hydroxyapatite has been linked to increased bone dissolution (49) and osteoclastic resorption (50), and related to fracture events in iliac crest biopsies (5). Interest in crystallinity has been long-standing because of previously reported tendency for hip fracture cases to develop some very large crystals (51) and the likelihood that such crystals are brittle (52,53). The inverse association between crystallinity and the carbonate-to-phosphate ratio present in the current study suggests a role for carbonate substitution in determining crystallinity, since the larger more perfect crystals, being more stable, should have a lower carbonate substitution (44). We expected higher carbonate content in the fracture group since this would reflect the presence of a less mature bone. We found instead that carbonate

substitution was significantly more homogeneous in the fracture group while mean levels were similar to controls. In contrast to previous FTIR imaging studies of osteoporotic iliac crest biopsies (54,55) no increase in mean bone crystal size was observed in the hip fracture cases, which is in agreement with recent results obtained by scanning small-angle X-ray scattering (32). Crystallinity, however, reflecting crystal size and perfection, was significantly more heterogeneous, suggesting the possibility that the wide size range seen may indicate an excess of brittle crystals in hip fractures with dimensions exceeding the postulated critical length of 30 nm (53).

Some clinical treatments may increase the variance of normal values for heterogeneity of FTIR parameters (mineral-to-matrix ratio, collagen maturity, carbonate-to-phosphate ratio, and crystallinity) measured in treatment-naïve cases of hip fracture. Osteoporotic patients treated with bisphosphonates, are likely to be affected by further reductions in heterogeneity. This decrease in bone heterogeneity, observed in dog tibias after 1 year's bisphosphonate treatment (15,20), was used to explain the significant accumulation of microdamage observed in vertebra of dogs given similar treatments (56,57). This association raises the possibility that reduced heterogeneity per se, in treatment or disease, is detrimental to bones' toughness. Healthy bone tissue is highly heterogeneous and there is evidence that this heterogeneity affects the mechanical properties of the bone material by preventing the proliferation of micro-cracks (15,22,23). Reduced heterogeneity at the nano- and macro-level is found in elderly patients (34), and reduced heterogeneity in modulus and hardness was also found in bisphosphonate treated patients with sub-trochanteric fractures (15), although the heterogeneity of plastic deformation resistance was increased in the same study. The beneficial effect of bisphosphonates in reducing remodeling by slowing the excessive removal of bone in osteoporotic patients (58-60), might be counterbalanced by the detrimental effects of limiting repair of micro-cracks and causing the development of excessive homogeneity, each of which, would lead the bone to become more brittle (61) and hence more likely to fracture. Therefore, bisphosphonates protect against fracture by maintaining bone mass. A loss of bone mass under the same amount of force would decrease bending resistance or increase the applied stress, leading to fracture. Changes in homogeneity and the decrease in repair of micro-cracks (62) might increase fracture risk despite a similar bone mass.

In conclusion, we have confirmed that hip fracture cases naïve to bisphosphonate therapy show reduced mineralization of their bone matrix compared to controls. This may contribute to their increased risk (43) of mechanically loading parts of the femoral neck beyond the so-called "yield point" at which damage-inducing plastic deformation takes place. In addition, hip fracture cases showed less heterogeneity of their mineral-to-matrix and carbonate-to-phosphate ratios, raising concerns that this could contribute to lower bone toughness leading to fracture. Also, there was a greater heterogeneity of crystallinity, which might increase the proportion of fragile crystals in fracture cases compared to the controls. Thus the mineral phase of femoral neck bone in hip fracture is different from control bone in several ways that are not captured by the clinical technique of bone densitometry. With aging, in contrast to osteoporosis, both mineralization and the carbonate-to-phosphate ratio increase progressively, alongside indentation modulus and hardness, at least in baboons (63). Further studies are needed to ascertain the effects of these differences on whole bone fragility and on how current and future treatments affect these abnormal properties of femoral neck bone in those at risk of hip fracture.

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Authors Responsibilities: Samuel Gourion-Arsiquaud performed the IR analysis and wrote and edited the first draft of the manuscript. Lyudmilla Lukashova performed and interpreted the micro-CT analysis and edited the paper. Jon Power prepared the embedded biopsy material, performed the light microscope histomorphometry and edited the manuscript. Nigel Loveridge and advised on the interpretation of the data edited the paper and with Jonathan Reeve wrote the non-NIH grants that supported collection and initial processing of the biopsies and light microscopy data. Jonathan Reeve also suggested the study and was responsible for the statistics. Adele Boskey wrote the grant that supported the study, coordinated the study performance, developed the study design and edited the final version of the study.

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References

1. de Laet CEDH, van Hout BA, Burger H, Hofman A, Pols HAP. Bone density and the risk of hip fracture in men and women: cross sectional analysis. *BMJ*. 1997; 315:221–225. [PubMed: 9253270]
2. Kanis JA, Odén A, McCloskey EV, Johansson H, Wahl DA, Cooper C. A systematic review of hip fracture incidence and probability of fracture worldwide. *Osteoporos Int*. Mar 15.2012 [Epub ahead of print].
3. Sánchez-Riera L, Wilson N, Kamalaraj N, Nolla JM, Kok C, Li Y, Macara M, Norman R, Chen JS, Smith EU, Sambrook PN, Hernández CS, Woolf A, March L. Osteoporosis and fragility fractures. *Best Pract Res Clin Rheumatol*. 2010; 24(6):793–810. [PubMed: 21665127]
4. McCready BR, Goldstein SA. Biomechanics of fracture: is bone mineral density sufficient to assess risk? *Journal of Bone & Mineral Research*. 2000; 15(12):2305–2308. [PubMed: 11127195]
5. Gourion-Arsiquaud S, Burket JC, Havill LM, DiCarlo E, Doty SB, Mendelsohn R, van der Meulen MC, Boskey AL. Spatial variation in osteonal bone properties relative to tissue and animal age. *J Bone Miner Res*. 2009; 24(7):1271–1281. [PubMed: 19210217]
6. Donnelly E. Methods for assessing bone quality: a review. *Clin Orthop Relat Res*. 2011; 469(8): 2128–2138. [PubMed: 21116752]
7. Black DM, Bouxsein ML, Marshall LM, Cummings SR, Lang TF, Cauley JA, Ensrud KE, Nielson CM, Orwoll ES, Osteoporotic Fractures in Men (MrOS) Research Group. Proximal femoral structure and the prediction of hip fracture in men: a large prospective study using QCT. *J Bone Miner Res*. 2008; 23(8):1326–1333. [PubMed: 18348697]
8. Kaptoge S, Beck TJ, Reeve J, Stone KL, Hillier TA, Cauley JA, Cummings SR. Prediction of incident hip fracture risk by femur geometry variables measured by hip structural analysis in the Study of Osteoporotic Fractures. *Journal of Bone & Mineral Research*. 2008; 23(12):1892–1904. [PubMed: 18684092]
9. Larrue A, Rattner A, Peter ZA, Olivier C, Laroche N, Vico L, Peyrin F. Synchrotron radiation micro-CT at the micrometer scale for the analysis of the three-dimensional morphology of microcracks in human trabecular bone. *PLoS One*. 2011; 6(7):e21297. [PubMed: 21750707]
10. Bell KL, Loveridge N, Power J, Stanton M, Meggitt BF, Reeve J. Regional differences in cortical porosity in the fractured femoral neck. *Bone*. 1999; 24(1):57–64. [PubMed: 9916785]
11. Bell GH, Dunbar O, Beck JS, Gibb A. Variations in strength of vertebrae with age and their relation to osteoporosis. *Calcif Tissue Res*. 1967; 1(1):75–86. [PubMed: 6060146]
12. Gupta HS, Seto J, Wagermaier W, Zaslansky P, Boesecke P, Fratzl P. Cooperative deformation of mineral and collagen in bone at the nanoscale. *Proc Natl Acad Sci U S A*. 2006; 103(47):17741–17746. [PubMed: 17095608]
13. Viguet-Carrin S, Garnero P, Delmas PD. The role of collagen in bone strength. *Osteoporos Int*. 2006; 17(3):319–336. [PubMed: 16341622]
14. Boskey AL, Mendelsohn R. Infrared spectroscopic characterization of mineralized tissues. *Vib Spectrosc*. 2005; 38(1-2):107–114. [PubMed: 16691288]

15. Donnelly E, Meredith DS, Nguyen JT, Gladnick BP, Rebolledo BJ, Shaffer AD, Lorch DG, Lane JM, Boskey AL. Reduced cortical bone compositional heterogeneity with bisphosphonate treatment in postmenopausal women with intertrochanteric and subtrochanteric fractures. *J Bone Miner Res.* 2012; 27(3):672–678. [PubMed: 22072397]
16. Gamsjaeger S, Buchinger B, Zwettler E, Recker R, Black D, Gasser JA, Eriksen EF, Klaushofer K, Paschalis EP. Bone material properties in actively bone-forming trabeculae in postmenopausal women with osteoporosis after three years of treatment with once-yearly Zoledronic acid. *J Bone Miner Res.* 2011; 26(1):12–18. [PubMed: 20645415]
17. Durchschlag E, Paschalis EP, Zoehrer R, Roschger P, Fratzl P, Recker RR, Phipps R, Klaushofer K. Bone material Properties in trabecular bone from human iliac crest biopsies after 3- and 5-year treatment with risedronate. *J Bone and Miner Res.* 2006; 21(10):1581–90. [PubMed: 16995813]
18. Fratzl P, Roschger P, Eschberger J, Abendroth B, Klaushofer K. Abnormal bone mineralization after fluoride treatment in osteoporosis: a small-angle x-ray-scattering study. *J Bone Miner Res.* 1994; 9(10):1541–1549. [PubMed: 7817799]
19. Röscher P, Rinnerthaler S, Yates J, Rodan GA, Fratzl P, Klaushofer K. Alendronate increases degree and uniformity of mineralisation in cancellous bone and decreases the porosity in cortical bone of osteoporotic women. *Bone.* 2001; 29(2):185–191. [PubMed: 11502482]
20. Gourion-Arsiquaud S, Allen MR, Burr DB, Vashishth D, Tang SY, Boskey AL. Bisphosphonate treatment modifies canine bone mineral and matrix properties and their heterogeneity. *Bone.* 2010; 46(3):666–672. [PubMed: 19925895]
21. Ciarelli TE, Fyhrie DP, Parfitt AM. Effects of vertebral bone fragility and bone formation rate on the mineralization levels of cancellous bone from white females. *Bone.* 2003; 32:311–315. [PubMed: 12667559]
22. Keaveny TM, Hayes WC. A 20-year perspective on the mechanical properties of trabecular bone. *J Biomech Eng.* 1993; 115:534–542. [PubMed: 8302037]
23. Renders GA, Mulder L, van Ruijven LJ, Langenbach GE, van Eijden TM. Mineral heterogeneity affects predictions of intratrabecular stress and strain. *J Biomech.* 2011; 44(3):402–407. [PubMed: 21040918]
24. Erben RG. Embedding of bone samples in methylmethacrylate: an improved method suitable for bone histomorphometry, histochemistry, and immunohistochemistry. *J Histochem Cytochem.* 1997; 45(2):307–313. [PubMed: 9016319]
25. Bell K, Loveridge N, Power J, Garrahan N, Stanton M, Lunt M, Meggitt B, Reeve J. Structure of the femoral neck in hip fracture: cortical bone loss in the inferoanterior to superoposterior axis. *Journal of Bone and Mineral Research.* 1999; 14:112–120.
26. Bell K, Loveridge N, Power J, Rushton N, Reeve J. Intracapsular hip fracture: Increased cortical remodelling in the thinned and porous anterior region of the femoral neck. *Osteoporosis International.* 1999; 10:248–257. [PubMed: 10525718]
27. Gourion-Arsiquaud S, West PA, Boskey AL. Fourier transform-infrared microspectroscopy and microscopic imaging. *Methods Mol Biol.* 2008; 455:293–303. [PubMed: 18463826]
28. Fritton JC, Myers ER, Wright TM, van der Meulen MC. Loading induces site-specific increases in mineral content assessed by microcomputed tomography of the mouse tibia. *Bone.* 2005; 36(6):1030–1038. [PubMed: 15878316]
29. Sedgwick P. Multiple significance tests: the Bonferroni correction. *British Medical Journal.* 2012; 344:e509.
30. Verdelis K, Ling Y, Sreenath T, Haruyama N, MacDougall M, van der Meulen MC, Lukashova L, Spevak L, Kulkarni AB, Boskey AL. DSPP effects on in vivo bone mineralization. *Bone.* 2008; 43(6):983–990. [PubMed: 18789408]
31. Loveridge N, Power J, Reeve J, Boyde A. Bone mineralization density and femoral neck fragility. *Bone.* 2004; 35(4):929–941. [PubMed: 15454100]
32. Fratzl-Zelman N, Roschger P, Gourrier A, Weber M, Misof BM, Loveridge N, Reeve J, Klaushofer K, Fratzl P. Combination of nanoindentation and quantitative backscattered electron imaging revealed altered bone material properties associated with femoral neck fragility. *Calcified Tissue International.* 2009; 85(4):335–343. [PubMed: 19756347]

33. Soicher MA, Wang X, Zauel RR, Fyhrie DP. Damage initiation sites in osteoporotic and normal human cancellous bone. *Bone*. 2011; 48(3):663–666. [PubMed: 21081188]
34. Zimmermann EA, Schaible E, Bale H, Barth HD, Tang SY, Reichert P, Busse B, Alliston T, Ager JW III, Ritchie RO. Age-related changes in the plasticity and toughness of human cortical bone at multiple length scales. *Proc Natl Acad Sci U S A*. 2011; 108(35):14416–14421. [PubMed: 21873221]
35. Busse B, Hahn M, Soltan M, Zustin J, Püschel K, Duda GN, Amling M. Increased calcium content and inhomogeneity of mineralization render bone toughness in osteoporosis: Mineralization, morphology and biomechanics of human single trabeculae. *Bone*. 2009; 45:1034–1043. [PubMed: 19679206]
36. Wand JS, Smith T, Green JR, Hesp R, Bradbeer JN, Reeve J. Whole body and site-specific bone remodelling in patients with previous femoral fractures. Relationships between reduced physical activity, reduced bone mass and increased bone resorption. *Clinical Science*. 1992; 83:665–675. [PubMed: 1336436]
37. Power J, Doube M, van Bezooijen R, Loveridge N, Reeve J. Osteocyte Recruitment declines as the Osteon fills in: Interacting Effects of Osteocytic Sclerostin and Previous Hip Fracture on the Size of Cortical Canals in the Femoral Neck. *Bone*. 2012; 50(5):1107–1114. [PubMed: 22353552]
38. Power J, Poole KE, van Bezooijen R, Doube M, Caballero-Alías AM, Lowik C, Papapoulos S, Reeve J, Loveridge N. Sclerostin and the regulation of bone formation: Effects in hip osteoarthritis and femoral neck fracture. *J Bone Miner Res*. 2010; 25(8):1867–1876. [PubMed: 20200987]
39. Ciarelli TE, Tjhia CK, Rao DS, Qiu S, Parfitt AM, Fyhrie DP. Trabecular packet-level lamellar density patterns differ by fracture status and bone formation rate in white females. *Bone*. 2009; 45:903–908. [PubMed: 19615479]
40. Fratzl P, Gupta HS, Fischer FD, Kolednik O. Hindered Crack Propagation in Materials with Periodically Varying Young's Modulus—Lessons from Biological Materials. *Advanced Materials*. 2007; 19(18):2657–2661.
41. Bousson V, Bergot C, Wu Y, Jolivet E, Zhou LQ, Laredo JD. Greater tissue mineralization heterogeneity in femoral neck cortex from hip-fractured females than controls. A microradiographic study. *Bone*. 2011; 48:1252–1259. [PubMed: 21397739]
42. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res*. 2010; 25(7):1468–1486. [PubMed: 20533309]
43. Thomas CDL, Mayhew PM, Power J, Poole KES, Loveridge N, Clement JG, Burgoyne CJ, Reeve J. Femoral Neck Trabecular Bone: Loss with Ageing and Role in Preventing Fracture. *J Bone and Miner Res*. 2009; 24:1808–18. [PubMed: 19419312]
44. Ruppel ME, Miller LM, Burr DB. The effect of the microscopic and nanoscale structure on bone fragility. *Osteoporosis International*. 2008; 19:1251–1265. [PubMed: 18317862]
45. Lotz JC, Cheal EJ, Hayes WC. Stress distributions within the proximal femur during gait and falls: implications for osteoporotic fracture. *Osteoporosis International*. 1995; 5(4):252–261. [PubMed: 7492864]
46. Rey C, Renugopalakrishnan V, Shimizu M, Collins B, Glimcher MJ. Fourier transform infrared spectroscopic study of the carbonate ions in bone mineral during aging. *Calcif Tissue Int*. 1991; 49(4):251–258. [PubMed: 1760769]
47. Tarnowski CP, Ignelzi MA Jr, Wang W, Taboas JM, Goldstein SA, Morris MD. Earliest mineral and matrix changes in force-induced musculoskeletal disease as revealed by Raman microspectroscopic imaging. *J Bone Miner Res*. 2004; 19(1):64–71. [PubMed: 14753738]
48. Kavukcuoglu NB, Patterson-Buckendahl P, Mann AB. Effect of osteocalcin deficiency on the nanomechanics and chemistry of mouse bones. *J Mech Behav Biomed Mater*. 2009; 2(4):348–354. [PubMed: 19627841]
49. LeGeros RZ, Kijkowska R, Bautista C, LeGeros JP. Synergistic effects of magnesium and carbonate on properties of biological and synthetic apatites. *Connect Tissue Res*. 1995; 33(1-3): 203–209. [PubMed: 7554956]

50. Doi Y, Iwanaga H, Shibutani T, Moriwaki Y, Iwayama Y. Osteoclastic responses to various calcium phosphates in cell cultures. *J Biomed Mater Res.* 1999; 47(3):424–433. [PubMed: 10487896]
51. Kent GN, Dodds RA, Klenerman L, Watts RW, Bitensky L, Chayen J. Changes in crystal size and orientation of acidic glycosaminoglycans at the fracture site in fractured necks of femur. *J Bone Joint Surg Br.* 1983; 65(2):189–94. [PubMed: 6826629]
52. Augat P, Schorlemmer S. The role of cortical bone and its microstructure in bone strength. *Age Ageing.* 2006; 35(Suppl 2):ii27–ii31. [PubMed: 16926200]
53. Gao H, Ji B, Jager IL, Arzt E, Fratzl P. Materials become insensitive to flaws at nanoscale: Lessons from nature. *Proc Natl Acad Sci U S A.* 2003; 100(10):5597–5600. [PubMed: 12732735]
54. Boskey AL, DiCarlo E, Paschalis EP, West P, Mendelsohn R. Comparison of mineral quality and quantity in iliac crest biopsies from high- and low-turnover osteoporosis: an FT-IR microspectroscopic investigation. *Osteoporos Int.* 2005; 16(12):2031–2038. [PubMed: 16088360]
55. Gourion-Arsiquaud S, Faibish D, Myers E, Spevak L, Compston JE, Hodsman A, Shane E, Recker RR, Boskey ER, Boskey AL. Use of FTIR spectroscopic imaging to identify parameters associated with fragility fracture. *J Bone Miner Res.* 2009; 24(9):1565–1571. [PubMed: 19419303]
56. Mashiba T, Hirano T, Turner CH, Forwood MR, Johnston CC, Burr DB. Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. *Journal of Bone & Mineral Research.* 2000; 15(4):613–620. [PubMed: 10780852]
57. Allen MR, Iwata K, Sato M, Burr DB. Raloxifene enhances vertebral mechanical properties independent of bone density. *Bone.* 2006; 39(5):1130–1135. [PubMed: 16814622]
58. Black DM, Schwartz AV, Ensrud KE, Cauley JA, Levis S, Quandt SA, Satterfield S, Wallace RB, Bauer DC, Palermo L, Wehren LE, Lombardi A, Santora AC, Cummings SR, FLEX Research Group. Effects of continuing or stopping alendronate after 5 years of treatment: the Fracture Intervention Trial Long-term Extension (FLEX): a randomized trial. *JAMA.* 2006; 296(24):2927–2938. [PubMed: 17190893]
59. Sebban A. Osteoporosis: how long should we treat? *Curr Opin Endocrinol Diabetes Obes.* 2008; 15(6):502–507. [PubMed: 18971678]
60. Geusens P. Bisphosphonates for postmenopausal osteoporosis: determining duration of treatment. *Curr Osteoporos Rep.* 2009; 7(1):12–17. [PubMed: 19239824]
61. Tjha CK, Odvina CV, Rao DS, Stover SM, Wang X, Fyhrie DP. Mechanical property and tissue mineral density differences among severely suppressed bone turnover (SSBT) patients, osteoporotic patients and normal subjects. *Bone.* 2011; 49:1279–1289. [PubMed: 21958843]
62. O'Neill JM, Diab T, Allen MR, Vidakovic B, Burr DB, Guldberg RE. One year of alendronate treatment lowers microstructural stresses associated with trabecular microdamage initiation. *Bone.* 2010; 47(2):241–247. [PubMed: 20483387]
63. Burket J, Gourion-Arsiquaud S, Havill LM, Baker SP, Boskey AL, van der Meulen MC. Microstructure and nanomechanical properties in osteons relate to tissue and animal age. *J Biomech.* 2011; 44(2):277–284. [PubMed: 21074774]

Fig. 1.
(left): Sketch of the cross-section of the femoral neck at mid-cortex. For the purposes of our study the sections were divided into four quadrants: Anterior, Superior, Posterior and Inferior (**From K. L. Bell, Bone 1999**). **(right)** Histogram of pixel values from a typical FTIR image to show the derivation of the FWHM (**F**ull **W**idth at **H**alf **M**aximum) parameter used to characterize FTIR heterogeneity (see text).

Fig. 2.

Box plot summaries of bone properties analyzed by micro CT. For both control (C) and fracture (F) groups, a) Tissue mineral density (TMD), b) bone volume fraction (BVF), c) Tissue mineral content (TMC) and d) Bone Volume (BV) were recorded in trabecular (Tb) and cortical (Cort) bone for each quadrant (Superior sup; Anterior ant; Inferior inf; and Posterior post) of the femoral neck biopsies.

Fig. 3.

(a): Typical Infrared images for the IR parameters recorded in inferior cortical quadrant from two patients, one with fractures and one without. Patient A had no fractures and was 85 years old at time of biopsy. The micro CT data for the cortical bone of this patient were: TMD=867, TMC=12.8, BVF=0.2 and Ct.Ar/Tt.Ar=0.42. Patient B had a hip fracture and was 86 years old at time of biopsy. The micro CT data for the cortical bone of this patient were: TMD=858, TMC=9.08, BVF=0.14 and Ct.Ar/Tt.Ar=0.55. Numerical values above the images are the means $\pm$ SEM for the parameter in the figure, and indicate the range of data for the pixels shown. 1 pixel = 6.25 microns.

(b): Variations in mean FTIR indices in trabecular and cortical bone of fracture cases and control subjects, averaged across all subjects in each group. Individual points refer to means for each quadrant and lines connect means averaged across all quadrants for fracture cases and controls separately. For statistical analyses of differences, see text.

(c): Variations in the logarithms of the FWHMs (heterogeneities) of the four FTIR indices. Displayed as for Fig 3b. For statistical analysis see text.

Fig. 4. Scatter plots of relationships between the means of the logarithms of the Carbonate: Phosphate ratio and Crystallinity for trabecular (dotted line) and cortical bone (solid line) respectively. Each point represents the mean result for one quadrant in one diagnostic group. The regression lines shown, with their 95% CI, are unadjusted for the effects of diagnosis, subject or anatomical quadrant. Triangles: cases of hip fracture; circles controls.

Table 1**2-D Morphometry of Femoral Neck Specimens**

	Cortical Thickness (25) mm*10		Cortical Porosity (10) %		Osteoid-bearing canals (26) %		Eroded canals (26) %	
	C	F	C	F	C	F	C	Y
Control/Fractures*								
Superior	15.5	12.0	10.1	11.2	5.8	8.3	1.2	3.0
Posterior	18.8	15.4	8.2	9.3	4.1	6.6	2.0	3.8
Anterior	23.0	19.5	10.9	11.9	3.7	6.2	1.8	3.6
Inferior	43.0	39.5	9.1	10.2	2.6	5.1	2.1	3.9

* Controls: mean age 80.4 years, range 68-89; fracture cases mean age 77.5 range 65-91 years. NB one control was not analyzed. When tested across all four quadrants (superior, posterior, anterior and inferior) mean differences between cases and controls in cortical thickness, cortical porosity, osteoid bearing canals and eroded canals were not significant at $p<0.05$, because of substantial within-group variability.

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Table 2

Micro-Computed Tomography of Fractured Femoral Necks and Age Matched Control Femoral Necks (mean $\pm$ SD)

	Control Cases (n=10)					Fracture Cases (n=10)				
	Cortical Bone Morphology									
Parameters	Total	Inferior	Superior	Anterior	Posterior	Total	Inferior	Superior	Anterior	Posterior
Tt.Ar	77 (±69)	86 (±80)	108 (±56)	17 (±27) <i>b,d,e</i>	96 (±71)	56 (±61)	53 (±62)	49 (±36) [#]	40 (±69)	80 (±36)
Ct.Ar	15 (±14)	31 (±16) <i>a,b,c</i>	10 (±7)	5.5 (±4)	15 (±13)	12 (±14)	22 (±19) <i>a,b</i>	7 (±3)	7 (±9)	13 (±14)
Ct.Ar/Tt.Ar	0.43 (±0.3)	0.63 (±0.3) <i>a,c</i>	0.107 (±0.06)	0.73 (±0.3) <i>d,e</i>	0.26 (±0.27)	0.44 (±0.3)	0.72 (±0.3) <i>a,c</i>	0.208 (±0.13)	0.509 (±0.31)	0.32 (±0.36)
Ct.Th	0.096 (±0.6)	1.9 (±0.72) <i>a,b,c</i>	0.54 (±0.14)	0.77 (±0.3)	0.63 (±0.13)	0.84 (±0.5)	1.7 (±0.6) <i>a,b,c</i>	0.56 (±0.14)	0.59 (±0.16)	0.57 (±0.12)
	Trabecular Bone Morphology									
BV/TV	0.12 (±0.07)	0.17 (±0.09) <i>a,b,c</i>	0.104 (±0.04)	0.102 (±0.04)	0.105 (±0.06)	0.1 (±0.04)	0.12 (±0.06)	0.1 (±0.03)	0.096 (±0.04)	0.09 (±0.02)
Tb.N	1.04 (±0.35)	1.14 (±0.44)	1.08 (±0.33)	0.93 (±0.31)	0.99 (±0.3)	1.04 (±0.35)	0.98 (±0.35)	0.99 (±0.3)	0.906 (±0.44)	0.97 (±0.27)
Tb.Th	0.11 (±0.03)	0.14 (±0.03) <i>a,b,c</i>	0.094 (±0.01)	0.106 (±0.01)	0.101 (±0.02)	0.11 (±0.03)	0.13 (±0.04) <i>a,b,c</i>	0.102 (±0.01)	0.11 (±0.04)	0.094 (±0.01)
Tb.Sp	0.97 (±0.4)	0.88 (±0.48)	0.9 (±0.29)	1.08 (±0.43)	1.02 (±0.42)	0.97 (±0.4)	1.06 (±0.54)	0.98 (±0.26)	1.2 (±0.51)	1.01 (±0.33)

^a p<0.05 (inf sup)

^b p<0.05 (inf ant)

^c p<0.05 (inf post)

^d p<0.05 (ant sup)

^e p<0.05 (ant post)

[#] p<0.05 (Control Fracture cases)

Tt.Ar Anterior quadrant lower than the other quadrants in control (p<0.05) not observed in fracture cases

Ct.Ar Inferior quadrant higher than the other quadrants in control (p<0.05) in fracture cases inf higher than sup and ant quadrants only.

Ct.Ar/Tt.Ar Inf and Ant are significantly different than the superior and posterior quadrants in control, in fracture cases the ant quadrant does not present any difference compare to the other quadrants.

Ct.Th Inferior quadrant higher than the other quadrants in control and fracture cases (p<0.05)

BV/TV Inferior quadrant higher than the other quadrants in control (p<0.05) not observed in fracture cases

Tb.Th Inferior quadrant higher than the other quadrants in control and fracture cases (p<0.05)